

Negative regulation of gene expression by the tumor suppressor p53

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ABSTRACT

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The tumor suppressor p53 inhibits the expression of a substantial number of genes whose protein products serve to promote cell survival or cell cycle progression, thereby ensuring efficient execution of p53-dependent apoptosis, cell-cycle arrest or senescence. Furthermore, p53-mediated repression has also been shown to participate in pathways that regulate diverse cellular processes, including angiogenesis, maintenance of pluripotency, and metabolic flux. p53 inhibits gene expression by both direct and indirect means. Briefly, p53 can block transcription through direct DNA binding, association with transcription factors, and through the induction of genes whose functional products facilitate downstream repression. Indirect regulation of gene repression by p53 often involves induction of intermediary factors that fall into several categories: proteins (e.g. p21), microRNAs (e.g. miR-34a), and lincRNAs (lincRNA-p21). This dissertation discusses multiple aspects of p53-dependent gene repression and presents novel targets of p53-mediated regulation. Specifically, we have found that p53 down-regulates the transcription of the oncogenic transcription factor FoxM1. Mechanistically, this repression is largely dependent upon the p53-inducible gene p21, and consequently involves the Rb-family of tumor suppressors. Functionally, p53-dependent repression of FoxM1 contributes to the maintenance of a stable G2 cell cycle arrest in response to DNA-damage. In addition, we have identified *PVT1* as a novel target of p53-transactivation. *PVT1* encodes both spliced non-coding RNAs (ncRNA), as

well as a series of microRNAs (miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p and miR-1208). p53 upregulates *PVT1* ncRNA, primary microRNAs, and mature miR-1204. Ectopic expression of miR-1204 induces changes in cell fate that are consistent with the role of p53 (cell death, cell cycle arrest), thus miR-1204 is likely to represent a functional target of p53 at the *PVT1* locus.

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Chapter 1

Introduction

Discovery and Historical Perspective

The *TP53* gene resides on chromosome 17p13.1 and encodes a 53 kilodalton protein, tumor protein p53, that is one of the most important proteins in cancer biology.

p53 was first identified in 1979 as a protein in complex with the large T antigen (LT) of SV40 polyomavirus (reviewed in (Braithwaite and Prives, 2006)). This discovery was made independently by two researchers, David Lane (Lane and Crawford, 1979) and Arnold Levine (Linzer and Levine, 1979). Seemingly consistent with the interaction of p53 and LT, p53 could be detected in multiple human cancer cell lines, but not normal human cells (Crawford et al., 1981). Furthermore, overexpression of cloned p53 cDNAs demonstrated that p53 contributes to cellular immortalization (Jenkins et al., 1984) and cooperates with the Ha-ras oncogene to transform normal cells (Eliyahu et al., 1984; Parada et al., 1984). Combined, these observations suggested that p53 acts an oncogene.

However, a landmark publication by Bert Vogelstein and colleagues in 1989 reported loss of heterozygosity (LOH) at the p53 locus in colorectal cancers (Baker et al., 1989). That is, mutations in the p53 gene were coupled with deletions at the p53 locus on the other allele. This suggested that inactivation of p53 is pro-tumorigenic, indicating that p53 is a tumor suppressor gene, not an oncogene. It was then realized that the cDNA clones of p53 that had been used in transformation assays were actually mutated versions of p53 and not the wild-type p53 sequence. Subsequent studies confirmed that wild-type p53 cDNA overexpression actually suppresses cellular transformation (Finlay et al.,

1989). These studies and other evidence (described below) clearly established p53 as a tumor suppressor.

After this shift in paradigm, an immense amount of work has gone into determining precisely how p53 may act to prevent tumor formation. Despite this, a comprehensive picture of p53 function has yet to emerge. In a most simplistic view, p53 is a transcription factor that is held at low, sometimes undetectable levels in normal cells, but is stabilized in response to cellular stress in order to facilitate anti-proliferative biological processes that prevent normal cells from transforming into cancer cells.

p53 and Cancer

Since the early 1990s, many observations have established p53 as one of the most important tumor suppressor proteins. p53 is one of the most frequently mutated genes in human cancer, with somatic mutations prevalent at many tumor sites (<http://www-p53.iarc.fr/>). Calculations of the mutation rate range from 5-80%, depending on the stage, etiology and type of tumor (Petitjean et al., 2007). Furthermore, cancer predisposition of patients with Li-Fraumeni syndrome is caused by germline mutation of p53 (Malkin et al., 1990; Srivastava et al., 1990). Experimentally, mice that have been genetically engineered to be devoid of the p53 protein spontaneously develop tumors at a much higher rate than normal mice. In fact, 75% of p53^{-/-} mice get tumors by the age of 6 months, as opposed to only 1% of wild-type mice developing tumors at 18 months (Donehower et al., 1992). Various mouse models have also shown that reactivation of wild-type p53 in an otherwise p53 null background causes regression of lymphomas,

sarcomas, as well as tumor regression in a liver carcinoma model (Christophorou et al., 2005; Christophorou et al., 2006; Kenzelmann Broz and Attardi, 2010; Xue et al., 2007).

In human tumors, most mutations of p53 result from small mutations- missense and nonsense mutations or insertions/deletions of several nucleotides (<http://p53.free.fr/index.html>)(Petitjean et al., 2007). 75% of observed p53 mutations in cancer arise from missense mutations, the substitution of a single amino-acid. The locations of such mutations within p53 tend to cluster in certain “hotspot” residues that occur very frequently in cancers. While different missense mutations perturb p53 function to different extents, there is a good correlation between complete loss of transactivation activity and occurrence in cancer (Petitjean et al., 2007).

Mutant forms of p53 not only lose functions that normally prevent tumorigenesis and tumor progression, but may also inhibit the activity of the remaining cellular wild-type p53 that is expressed from the other allele. Since p53 functions as a tetramer, the mutant p53 is able to function as a dominant-negative simply by complexing with the wild-type protein (Oren and Rotter, 2010).

Furthermore, it has been proposed that mutant p53 also acquires new functions which contribute to tumorigenic processes. The observations in human tumors that led to this hypothesis were the very high levels to which mutant p53 accumulates in cancer cells (Oren and Rotter, 2010) and the frequency of LOH, suggesting that cancer cells acquire a selective advantage by losing the remaining wild-type p53 (reviewed in (Brosh and Rotter, 2009)). In an experimental setting, mutant p53 overexpression in a p53 null background was shown to transform cells, allowing them to grow in soft-agar and form tumors in mice (Dittmer et al., 1993). Subsequent studies using both cell culture and

animal models have established mutant p53 “gain-of-function” roles in promoting genomic instability, preventing apoptosis, enhancing cell migration and invasion and augmenting cell proliferation, among others (Reviewed in (Oren and Rotter, 2010)).

In addition to p53 mutations, the activity of wild-type p53 can also be suppressed in cancer cells by various mechanisms. Many DNA tumor viruses encode proteins that target p53. For example, SV40 large tumor antigen, Adenovirus E1B protein, and human papilloma virus E6 protein all bind and inactivate p53 (reviewed in (Vousden, 1995) and (Levine, 2009)). In certain cases of inflammatory breast cancer and neuroblastoma, p53 is excluded from the nucleus and thus is unable to function as a transcription factor (Moll et al., 1995; Moll et al., 1996; Moll et al., 1992).

Overexpression of cellular factors that inhibit p53 also render p53 inactive in tumors. The major negative regulator of p53 stability and activity is the oncoprotein Mdm2 (reviewed in (Iwakuma and Lozano, 2003; Moll and Petrenko, 2003)). Mdm2 is often overexpressed in certain cancer types, especially sarcomas (reviewed in (Onel and Cordon-Cardo, 2004)). A highly homologous protein, Mdmx, also serves as a negative regulator of p53, and is overexpressed in many human cancers (reviewed in (Marine et al., 2007; Toledo and Wahl, 2007)). Alterations in the PTEN and Akt pathways also increase nuclear Mdm2 and impair p53 function (<http://p53.free.fr/index.html>). Finally, defects in upstream signaling pathways can also impair the response of p53 to cellular stress. Upon cellular stressors such as DNA damage, proteins such as ATM and Chk2 phosphorylate p53, disrupting its interaction with Mdm2 and thus activate p53 (Caspari, 2000). Mutations in this pathway, or others that similarly modify p53, may also affect the p53-response.

p53: the basic facts

p53 is a transcription factor that regulates a plethora of downstream target genes. The p53 protein, like other transcription factors, harbors several distinct regions including two transactivation domains, a core sequence specific DNA-binding domain, an oligomerization domain, and a C-terminal basic domain. These regions of p53 function in a coordinated fashion to integrate upstream signaling resulting from a wide variety of cellular stressors (e.g. DNA-damage, hypoxia, replication stress, oncogenic stress, metabolic changes, growth factors, cytokines). Through transcriptional regulation of downstream target genes in response to stress, p53 initiates cytoprotective or adaptive processes such as cell cycle arrest or cell death, among others (reviewed in (Prives and Hall, 1999)).

Most likely owing to the severity of the p53-response, p53 is regulated by multiple cellular mechanisms. Mdm2 is the major negative regulator of p53. Mdm2 binds to p53 and inhibits its ability to transactivate target genes. Mdm2 also serves as the major E3 ubiquitin ligase for p53, targeting p53 for degradation and facilitating its transport to the proteasome (reviewed in (Moll and Petrenko, 2003)). A homologous protein, Mdmx, also binds p53 and inactivates p53's transcriptional activity. Unlike Mdm2, however, Mdmx does not act as a ubiquitin ligase for p53 (Marine and Jochemsen, 2005; Toledo and Wahl, 2007).

p53 is also modified post-translationally by phosphorylation, acetylation, methylation, sumoylation, neddylation and ubiquitination. Certain modifications, such as phosphorylation of Ser15, Thr18 and Ser20 are thought to disrupt the p53-Mdm2 interaction, thereby stabilizing and activating p53 (Shieh et al., 1997) ,(reviewed in

(Olsson et al., 2007)). Phosphorylation (e.g. at ser46), or other modification of distinct residues are thought to mediate promoter selectivity - that is, alterations in which subset of p53 target genes are activated in response to a given stress (reviewed in (Vousden and Prives, 2009)). Finally, p53 interacts with many additional cellular factors that direct p53 activity toward different cellular outcomes. For example, p53 interaction with the ankyrin-repeat-, SH3-domain- and proline-rich-region-containing protein (ASPP) family directs p53 activity specifically toward cell death and not cell cycle arrest (reviewed in (Trigiante and Lu, 2006)).

p53 the transcription factor

Though multiple functions of p53 have been described, p53 is most well-characterized as a transcription factor. To function as such, p53 forms a tetramer of identical subunits. Of particular importance to p53's transcriptional activity, each monomer includes transactivation domains and DNA-binding domains. These two types of domains account for the major functions which need to be accomplished by transcription factors: DNA-binding and co-factor recruitment (Laptenko and Prives, 2006).

The core DNA-binding domain of p53 is folded in such a way that results in sequence specific DNA binding. Evidence of the importance of this domain to p53 function is that the majority of p53 mutations found in cancer alter residues that directly contact DNA or disrupt overall conformation of this domain (Prives and Hall, 1999). The C-terminal domain, on the other hand, contacts DNA non-specifically (reviewed in (Jayaraman and Prives, 1999; Kim and Deppert, 2006; Liu and Kulesz-Martin, 2006),

allowing p53 to diffuse linearly along DNA (McKinney et al., 2004), potentially allowing the core domain to recognize a specific sequence, or response element (RE).

Transcriptional Activation

In order to directly transactivate genes, p53 recognizes and binds a RE that is located in close proximity to a target gene. Target genes that are robustly induced by p53 often contain at least one RE within a few thousand base pairs of the transcription start site (TSS), and may also contain multiple binding sites spaced far from one another (Laptenko and Prives, 2006). The RE is often located in the upstream promoter region of target genes, but can also be found very close to the TSS, in intronic regions or even exonic regions (reviewed in (Beckerman and Prives, 2010)).

The canonical RE consists of two half sites separated by a spacer of variable length (Reviewed in (Riley et al., 2008)). Each half-site consists of two pentameric quarter-sites that are often arranged head-to-head. The degenerate consensus sequence to which p53 has been shown to bind with high affinity is as follows: 5'-RRRCWWGYYY-3' (R is a purine, Y a pyrimidine, W is either Adenine (A) or Thymine (T), C is Cytosine and G is Guanine) (el-Deiry et al., 1992; Funk et al., 1992; Hoh et al., 2002). This sequence, 5'-RRRCWWGYYY-3', represents one half-site. The canonical p53 RE consists of two of these half-sites separated by a spacer of 0-21 nucleotides (Riley et al., 2008). In certain cases, p53 has been shown to recognize non-canonical REs. For example, some p53-responsive elements contain more than two-half sites (e.g. AQP3 (Zheng and Chen, 2001)), or have a different orientation of the pentameric sites (e.g. MDR1 (Johnson et al., 2001)), or are comprised of a totally different structure such as the

pentanucleotide motif TGYCC found in the microsatellite region of PIG3 (Contente et al., 2002).

Once p53 has recognized and bound to a RE within the DNA of a target gene, p53 is then able to facilitate either transcriptional activation or repression at this locus. The p53 N-terminus is an unstructured domain belonging to the class of ‘acidic’ activation domains (Laptenko and Prives, 2006) that are able to interact with several factors of the general transcription machinery (Ko and Prives, 1996). In fact, the interaction of the transactivation domain (TAD) with TATA-binding protein (TBP) correlates with p53 transactivation ability (Chang et al., 1995).

Several regions of the N-terminus contribute to the transcriptional activity of p53. Two regions that are essential for full transcriptional activity span from residues 1–42 and 43–73 (Chang et al., 1995). Owing to the importance of the first region, mutations of two hydrophobic residues, Leu22 and Trp23, greatly diminishes transactivation by p53 (Lin et al., 1994), but does not abolish the activity completely (Baptiste-Okoh et al., 2008b; Scoumanne et al., 2005). Mutation of two residues from the second domain, Trp53 and Phe54, also impair the transactivation of certain p53-targets (Candau et al., 1997; Zhu et al., 1998). Other regions, such as the proline rich region (residues 63–97) have also been shown to contribute to p53’s transcriptional activity (Dornan et al., 2003; Zacchi et al., 2002; Zheng et al., 2002).

The central function of the p53 TAD is to recruit and interact with factors that mediate eventual transcription of the target gene by RNA Polymerase II. To achieve transcription initiation, histones in the promoter region must be modified in such a way that promotes the open configuration of chromatin. This open configuration then allows

members of the general transcription machinery to bind to DNA and facilitate transcription of RNA. p53 interacts with multiple proteins that contribute to open chromatin configurations (reviewed in (Beckerman and Prives, 2010)). For example, p53 interacts with chromatin remodeling factors such as the SWI/SNF complex (Lee et al., 2002a), histone acetyltransferases (HAT) such as p300 (Avantaggiati et al., 1997; Gu and Roeder, 1997; Lill et al., 1997; Scolnick et al., 1997), pCAF (Scolnick et al., 1997) (Barlev et al., 2001), GCN5 (Candau et al., 1997), and TIP60 (Gevry et al., 2007). Further, p53 serves to recruit additional factors such as histone variant H2A.Z (Gevry et al., 2007) and the arginine methyltransferases PRMT1 and CARM1 (An et al., 2004) to certain promoters to facilitate full transcriptional activity.

After remodeling of the chromatin, members of the preinitiation complex are recruited or altered in some fashion (reviewed in (Beckerman and Prives, 2010)). The preinitiation complex consists of factors such as TFIID (TBP), TFIIB, and other general transcription factors (TFIIF, TFIIE, TFIIH) that form a complex with RNA Polymerase II. In addition to recruiting chromatin remodeling factors, p53 also plays a role in the recruitment of the preinitiation complex. For example, p53 interacts with TBP and associated factors (Chen et al., 1993; Farmer et al., 1996a; Liu et al., 1993; Thut et al., 1995), as well as TFIIA and TFIIH (Xing et al., 2001), (reviewed in (Ko and Prives, 1996)).

Transcriptional Repression

While most studies of p53 have focused on its ability to activate transcription, p53 also serves as a transcriptional repressor. In fact, global analysis of gene expression

changes upon p53 activation has revealed that p53 represses a significant number of genes (Burns and El-Deiry, 2003; Mirza et al., 2003; Robinson et al., 2003; Sax et al., 2003). p53 may repress genes either directly (through direct interactions between p53 and DNA) or indirectly.

The idea of a “dual-acting” transcription factor that both activates and represses transcription is well established, and an early example of such a factor is the thyroid hormone receptor. In the absence of the thyroid hormone, thyroid hormone receptor binds the promoters of genes which contain its binding site and represses transcription through recruitment of N-CoR and mSin3. When the receptor binds the thyroid hormone, it changes conformation and binds coactivator molecules such as CREB binding protein (CBP), and activates transcription (reviewed in (Latchman, 2001)). p53, in contrast, does not shift between conformations based on hormone binding. However, post-translational modification of p53 upon diverse cellular stressors may affect p53 conformation and p53-binding partners, converting p53 from an activator to a repressor.

Another example of a ‘dual’-acting transcription factor is the glucocorticoid receptor. This receptor binds DNA as a consequence of hormone treatment. When bound to a glucocorticoid response element (GRE), the receptor activates transcription. However, the receptor also binds genes with a related response element known as nGRE. At these genes, the glucocorticoid receptor represses transcription (reviewed in (Latchman, 2001)). Yet another example of this phenomenon comes from the Pit-1 transcription factor. The presence of two additional thymine bases in the Pit-1 binding site alters the DNA-bound conformation of Pit-1 in such a way that allows recruitment of the co-repressor N-CoR, resulting transcriptional repression instead of activation (Scully

et al., 2000). Thus, the DNA sequence of the response element can act as a determinant for activation or repression by a particular transcription factor.

Direct Transcriptional Repression mediated by p53

This situation is also applicable for the p53 ‘dual’-acting transcription factor. The MDR1 locus contains a p53 RE that has an opposite orientation from the canonical RE. That is, the pentameric quarter sites are arranged in a head-to-tail fashion, rather than the usual head-to-head fashion. When bound to this site, p53 represses MDR1 transcription. However, p53 activates an MDR1 construct in which the orientation of the RE was artificially transformed into the head-to-head arrangement (Johnson et al., 2001). Furthermore, the p53 repressed gene CD44 contains a p53 RE in with strong sequence similarity to the MDR1 site. This site, which p53 binds directly and specifically, is necessary for p53-mediated repression of CD44. These studies suggest that the orientation of the p53 RE affects the conformation with which p53 binds to DNA, and ultimately what co-factors p53 may recruit to the promoter to activate or repress transcription.

The idea that alterations of the canonical p53 RE can transform p53 from an activator into a transcriptional repressor is not confined to changes in orientation of the response element as seen in the MDR1 and CD44 promoters (reviewed in (Wang et al., 2010)). A recent systematic study on the role of specific nucleotides of the p53 RE revealed that a slight sequence alteration in the core RE to be a key determinant in p53’s decision to activate or repress a target gene (Wang et al., 2009). Through comparison of the Lasp1 and p21 p53-REs, the authors identify a p53 repression response element:

RRXCXXGXYX-XXCXXGXYX (X can be A, C, G or T). This sequence differs from the canonical half-site (RRRCWWGYYY) in that the core nucleotides between the invariant C and G are not restricted to be A or T. In addition, there are often variations in the nucleotides flanking the core sequence. Using this sequence, the authors successfully predicted the transcriptional outcome facilitated by many published REs (verified experimentally) (Wang et al., 2009). As such, it may be possible to re-evaluate repression targets that have up until now considered indirect due to lack of a canonical p53 RE (Wang et al., 2010).

How does the nature of the p53 response element determine whether p53 activates or represses a target gene? As described above, DNA-bound p53 interacts with a variety of factors that remodel chromatin and initiate transcription. Repressive REs, on the other hand, may cause p53 to bind DNA with a different conformation. If alternative surfaces of p53 were exposed at p53-repressive REs, p53 may interact with and recruit a different subset of co-factors that facilitate repression instead of activation.

In line with this hypothesis, p53 directly interacts with the co-repressor mSin3a, and through this interaction recruits histone deacetylases (HDAC) to target genes (Murphy et al., 1999). The mSin3-HDAC complex deacetylates core histones, promoting a closed, or repressed, chromatin state (Grzenda et al., 2009). Map4 was the first p53-repressed gene reported to be downregulated by this mechanism. Despite the lack of a canonical p53-RE, p53 does bind to the Map4 locus (Murphy et al., 1999). Similarly, p53 interacts with the Mad1 promoter through a non-canonical site, facilitating mSin3a-HDAC recruitment (Chun and Jin, 2003). p53 also recruits the mSin3a-HDAC to p53-repressed genes that contain binding sites that more closely resemble the canonical p53

RE. For example, p53 was shown to interact both by chromatin immunoprecipitation analysis (ChIP) and electrophoretic mobility shift assays (EMSA) at REs in *Nanog* (Lin et al., 2005) and *Hsp90beta* (Zhang et al., 2004). Another gene to which p53 targets the mSin3-HDAC complex is *c-Myc*, which contains multiple regions of p53 interaction, some which include the canonical RE and others that lack a RE (Ho et al., 2005).

Through the examples detailed above, it has become evident that p53 facilitates chromatin remodeling in such a way that leads to repression of target genes. Less clear is the exact mechanism that leads to interaction between p53 and co-repressors (instead of co-activators) at such target genes. The genes described above may in fact contain previously unidentified repressive RE, and these sites may facilitate direct p53 binding in a conformation that promotes recruitment of co-repressors. Alternatively, p53 may interact with the DNA through other transcription factors (discussed below), rendering its conformation amenable to interactions with co-repressors. In at least one case, however, a direct interaction between p53 and DNA was observed- the *Nanog* canonical p53 RE (Lin et al., 2005). Here, the authors propose that p53 represses transcription from this RE due to spacer length between p53 half-sites. This is consistent with earlier reports demonstrating that deletion of a 3-bp spacer from the *Survivin* p53 RE converted the site from a repressive to activating site (Hoffman et al., 2002). However, more recent data suggest that the effect of spacer length is highly variable (reviewed in (Wang et al., 2010)).

Another factor that may contribute to p53's decision to activate or repress these loci, and others, is the type stimulus that leads to p53 activation. Due to extensive cross-talk between p53 and multiple cellular pathways, different cellular stresses lead to

different p53-dependent biological outcomes. Both post-translational modifications and interacting protein partners influence p53 activity. In certain documented cases, the type of cellular stress determines whether p53 predominantly acts as a transcriptional activator or repressor. For example, hypoxia is a stress that leads to broad transcriptional repression by p53, but only selective transactivation (Hammond et al., 2006).

Mechanistically, hypoxia promotes the interaction between p53 and mSin3, but not the interaction between p53 and the HAT p300. In contrast, DNA-damaging agents promote both such interactions (Koumenis et al., 2001). After stresses such as hypoxia, the modification status of p53 and additional factors (e.g. promoter architecture, other transcription factors) undoubtedly contribute to p53's transrepression capabilities.

In addition to interaction with co-repressors, p53 can inhibit transcription by a variety of mechanisms. Some of these mechanisms involve the interaction of p53 with a p53 RE at the repressed target, and some do not. In general, these two overall modes of repression have been classified into direct and indirect repression, though the distinction between the categories can sometimes be blurred. As discussed above, clear examples of direct repression are those which involve p53 direct interaction with a RE (e.g. a repressive RE like found at MDR1) that facilitates p53-mediated recruitment of repressive complexes. The identification and refinement of a consensus repressive RE will greatly increase our understanding of the inherent ability of p53 to directly repress transcription.

“Activator occlusion” represents another flavor of direct repression, and has been described as the mechanism of repression at select target genes. In this scenario, a canonical p53 RE is located in close proximity to, or overlaps, the response element of

another transcription factor. When p53 binds to its canonical RE, it displaces the other transcription factor. This promoter bound-p53 may still activate transcription, but due to displacement of a more potent transcription factor, a net repression is observed (reviewed in (Johnson et al., 2001)). For example, p53 represses the alpha-fetoprotein (AFP) gene by direct binding that competes with binding of the transactivator hepatic nuclear factor 3 (HNF-3). In cells that lack HNF-3, p53 indeed activates transcription through this site (Lee et al., 1999). Other examples of this mechanism are found at the DNA polymerase δ catalytic subunit gene (POLD1) gene, (where p53 binding displaces the Sp1 transcription factor) (Li and Lee, 2001), the CDC25C gene (where p53 binding overlaps a GC box) (St Clair et al., 2004), and the HBV gene (where p53 binds adjacent to an enhancer region) (Ori et al., 1998). In an additional case, p53 and the activator Brn-3 bind to adjacent sites in the p53-repressed target Bcl-2. However, the physical interaction between p53 and Brn-3 complicates the mechanism operating at this promoter (Budhram-Mahadeo et al., 1999; Ho and Benchimol, 2003).

Direct/Indirect Repression:

Another subset of genes is repressed by the interaction of p53 and upstream activators, and blurs the distinction between direct and indirect repression. In this mode of repression, p53 does not directly contact the DNA of the repressed promoter. Yet in some cases, p53 can still localize to the promoter of the repressed gene due to such interactions. For example, p53 binds to trimers of the NF-Y transcription factor following DNA-damage, and through this binding is tethered to the promoters of genes such as cyclin B2, Cdc25C, and Cdc2 (Imbriano et al., 2005). At these genes, p53

recruits HDAC complexes to inhibit transcription. Consistent with the premise that modifications of p53 contribute to transcriptional repression, these studies found that the C-terminal lysines of p53 (targets of both acetylation and methylation), are essential for recruitment of HDAC4 and downstream repression (Basile et al., 2006). In addition, p53 has been found to interact with and repress transcription by binding to Sp1 (at the hTERT (Kanaya et al., 2000), IGF-1 (Ohlsson et al., 1998) , CDC25B (Dalvai et al., 2011), Cyclin B1 (Innocente and Lee, 2005) and DNMT1 (Lin et al., 2010) promoters), ets-1 (at the TXSA (Kim et al., 2003) and IKK α promoters (Gu et al., 2004)), C/EBP β (at the albumin promoter (Kubicka et al., 1999), and AP-1 (at the hMMP-1 promoter (Sun et al., 1999))

In other cases, p53 interacts with transcriptional activators and prevents them from binding the promoter. For example, repression of the eukaryotic initiation factor 4E (eIF4E) involves p53 binding to the c-Myc transcription factor, an interaction which inhibits the ability of c-Myc to bind and stimulate the eIF4E promoter (Zhu et al., 2005). Similarly, p53 interacts with the estrogen receptor, preventing its interaction with the estrogen response element (Liu et al., 1999).

Indirect Repression

Indirect p53-mediated repression refers to cases in which p53 downregulates transcription without directly and specifically contacting DNA. Similar to the multiple modes of direct repression facilitated by p53, indirect repression can also take on many forms such as squelching of the general transcription machinery, inhibition of specific

transcription factors (as discussed above), and transactivation of factors that act as downstream transcriptional repressors.

Experiments conducted in the early to mid-1990s provided the first insights into p53's ability to repress transcription. Collectively, these observations suggested that high levels of p53 squelch activated transcription through titration of molecules necessary for transcriptional initiation. For example, p53 was shown to repress TATA box containing genes, but not genes whose transcription relies on a pyrimidine-rich initiator element (Mack et al., 1993). This repression was shown to be mediated by the interaction of p53 with the basal transcription machinery (Mack et al., 1993). However, further studies showed that the p53 interaction with the TATA-binding protein itself was dispensable for interference with transcriptional initiation (Farmer et al., 1996b), suggesting that p53 interaction with other transcription-associated factors (TAFs) such as TFIIB and TFIID mediated this effect (Liu and Berk, 1995). While these studies provided much insight into p53 function and binding partners, the overexpression of p53 to non-physiological levels in these experiments complicates their interpretation.

Given the observation that stabilization of endogenous cellular p53 does in fact lead to the repression of a multitude of genes, multiple mechanisms were proposed to explain how physiological levels of p53 could inhibit transcription. Many of these mechanisms, such as the direct binding of p53 to its consensus binding elements, were discussed above. Yet, given the well established role of p53 as a transcriptional activator, an alternative hypothesis was proposed. This hypothesis suggested that p53 may increase the level of another transcriptional regulator that negatively regulates downstream transcriptional activity. In 2003, elegant work from the Dobbstein lab confirmed this

hypothesis by showing the importance of p21/CDKN1A to p53-mediated repression (Lohr et al., 2003).

Indirect repression: p21/CDKN1A

One of the first p53-responsive genes to be identified was the cyclin dependent kinase inhibitor p21/CDKN1A (el-Deiry et al., 1993). The protein product of this gene is a key p53-effector, inhibiting progress through the cell cycle by interference with the activity of cyclin-dependent kinases (CDK) (Boulaire et al., 2000). This inhibition of CDK activity serves to prevent the phosphorylation of another class of tumor suppressor proteins, the Retinoblastoma (Rb) family (p130, p107 and p105RB). When hypophosphorylated, the Rb-family proteins bind transcription factors E2F1-5, converting such factors from transcriptional activators to repressors (Bartek and Lukas, 2001). These findings, coupled with the observations that an engineered mutant of p53 (p53 mutated at residues 22 and 23) abolished both activation and repression by p53 (Lin et al., 1994; Murphy et al., 1996), led the authors to pursue the idea that p53-mediated transcriptional repression is mediated primarily by p21 (Lohr et al., 2003). In fact, two genes whose protein products participate in the G2/M checkpoint, Chk1 and cdc2, had previously been shown to be downregulated by p53 and the p21/pRb/E2F axis (Flatt et al., 2000; Gottifredi et al., 2001; Taylor et al., 2001). To assess the general dependence on p21 for p53-mediated repression, Lohr et al (2003) employed HCT116 colon carcinoma cell lines that contain targeted disruptions of the p21 locus. In these cells, the p53-mediated repression of all genes tested (Survivin, Stathmin, Cyclin B1, Topoisomerase 2 alpha, Chk1, Cdc25c, Cdc2, Brca1, Cyclin A2, Pold1, Lbr) was

completely abrogated in the absence of p21. These data, combined with the observation that p21 overexpression alone caused repression of most of these genes, suggest that p21 is necessary as well as sufficient for mediating a subset of p53-dependent transcriptional repression (Lohr et al., 2003).

Consistent with this idea, additional work has further delineated the role of and importance of the p21/Rb-family/E2F pathway to p53-mediated repression. For example, Rb-family-null MEFs are unable to repress the p53-repression target Plk1 (Jackson et al., 2005). Additional genes have also been shown to require this pathway for their repression, including but not limited to hTERT (Shats et al., 2004), MCM-7 (Scian et al., 2008) and Caspase-2 (Baptiste-Okoh et al., 2008a).

Mechanistically, p53/p21 activation has been shown to result in binding of the Rb/E2F complex to specific promoter elements of the repressed gene. For example, overexpression of p53 leads to binding of p130/E2F4 to the R box of the Cdc2 promoter (Taylor et al., 2001). On the other hand, the Plk1 gene contains a CDE/CHR element that contributes to its repression by p21 (Zhu et al., 2002). The p53-dependent repression of Cdc25C was also shown to be at least partially dependent on a CDE/CHR element (St Clair et al., 2004). CDE sites are a special class of E2F-binding site that require a CDR element four nucleotides downstream of the CDE site. Like Plk1, gene promoters that contain CDE/CHR elements often encode proteins that function in cell cycle progression (specifically S, G2, and M phases) (Muller and Engeland, 2010). Intriguingly, the occurrence of this promoter element seems to correlate with genes that are repressed by both DNA-damage activated p53 (Badie et al., 2000) as well as p130/p107 (Jackson et al., 2005). For example, the p53-repression targets Cdc2, Cyclin B1, Cyclin B2, and

Cdc25C (de Toledo et al., 1998; Haugwitz et al., 2002; Innocente et al., 1999; Krause et al., 2001; Krause et al., 2000; Taylor et al., 1999b) all contain CDE/CHR elements. In contrast, other p53-repressed genes such as Cdc25A (Rother et al., 2007b) and Cks2 (Rother et al., 2007a) do not contain such elements. Despite the correlation between p53-repressed genes and the presence of CDE/CHR promoter elements, the importance of CDE/CHR elements to p53-mediated repression is still unclear due to conflicting reports and partial dependencies on such elements. Yet, one recent report describes a p53-dependent switch in a putative complex that binds the CDE/CHR binding site (the DREAM complex) (Muller and Engeland, 2010). Upon p53 activation, the complex is converted from a transcriptional activator (containing b-Myb) to a transcriptional repressor (containing p130/E2F4) (Mannefeld et al., 2009), suggesting that the CDE/CHR elements may indeed play a specific role in p53-mediated repression.

Indirect repression: Non-coding RNA

While p21 is indeed important for the execution of transcriptional repression downstream of p53, recent reports have described a new class of p53 inducible targets that also act by negatively regulating gene expression: non-coding RNA.

Non-coding RNAs, as the name implies, are RNA molecules that do not encode for protein products. Some familiar classes of non-coding RNAs are ribosomal RNA and transfer RNA. These well studied RNAs function together within the ribosome, interacting to ensure that each messenger RNA codon is translated into the addition of the proper amino acid in a growing amino acid chain. However, many other classes of non-coding RNA also exist within the cell, including small nuclear RNA (snRNA)(Mattaj et

al., 1993), small nucleolar RNA (snoRNA) (Bachellerie et al., 2002; Mattaj et al., 1993), small interfering RNA (siRNA) (Elbashir et al., 2001; Hamilton and Baulcombe, 1999), piwi interacting RNA (piRNA) (Aravin et al., 2007), microRNA (miRNA) (Bartel, 2009) and long non-coding RNA (lncRNA) (Ponting et al., 2009). It is becoming increasingly clear that such classes of RNA, in particular microRNAs and lncRNAs, are heavily involved in the regulation of gene expression, both at the transcriptional and post-transcriptional levels (Khalil and Rinn, 2011; Ponting et al., 2009).

microRNAs

microRNAs are small RNA molecules of approximately 19-25 nucleotides that target messenger RNAs (mRNA) for degradation or inhibition of translation (Bartel, 2004). The discovery of this class of RNA molecules came from developmental studies in the worm *Caenorhabditis elegans*. These studies revealed two temporally expressed small regulatory RNAs, lin-4 (Lee et al., 1993) and let-7 (Reinhart et al., 2000), which were later determined to represent an abundant class of endogenous RNAs expressed across diverse species (Griffiths-Jones et al., 2008; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Distinguishing microRNAs from other classes small RNAs are the characteristic hairpin structure from which the final mature microRNA is derived (Bartel, 2009).

The biogenesis of microRNAs begins with transcription of primary microRNA transcripts (pri-miRs) that are transcribed by RNA polymerase II, capped and polyadenylated (Ambros, 2004; Bartel, 2004; Cai et al., 2004; He and Hannon, 2004).

This transcript is cleaved by the nuclear RNase III endonuclease Drosha, freeing a 60-70 nucleotide stem-loop structure, or pre-miRNA (Lee et al., 2002b; Zeng and Cullen, 2003). This precursor is processed in such a way that generates a 5' phosphate and a ~2 nucleotide 3' overhang (Basyuk et al., 2003; Lee et al., 2003). The pre-miRNA is subsequently exported from the nucleus by Exportin-5 and GTP-bound Ran (Lund et al., 2004; Yi et al., 2003). Cytoplasmic Dicer then cleaves both strands of the pre-miRNA duplex, cutting off the overhang and loop portion of the stem-loop structure, leaving an siRNA-like imperfectly matched double-stranded RNA molecule. One strand of this duplex represents the mature microRNA, which is subsequently incorporated into the RNA-induced silencing complex (RISC). Within the RISC complex, the mature microRNA hybridizes to sequences found within a target mRNA. Two outcomes can result from this pairing: degradation of the mRNA, or inhibition of its translation. This choice is thought to be predominantly decided by the extent of complementarity between the miRNA and the mRNA sequence, with the higher percentage of complementary pairings leading to cleavage (Reviewed in (Bartel, 2004)). Regardless of the exact mechanism by which microRNAs inhibit their targets, microRNAs have the potential to be a very powerful class of regulators due to their ability to target a large number of cellular mRNAs (Lim et al., 2005) .

In 2007, four separate publications independently identified miR-34a as a transcriptional target of p53, linking the tumor suppressor p53 to the microRNA world (Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tazawa et al., 2007). Activated p53 binds to a canonical binding site within the miR-34 gene, ~ 30 kilobase pairs (kb) upstream of the mature miR-34a, inducing the transcription of the primary

transcript. Production of the mature miR-34a, however, requires removal of a 30 kb intron (Raver-Shapira et al., 2007). Once in its mature form, miR-34a greatly influences gene expression patterns. Importantly, genes downregulated by miR-34a expression fall into categories of genes normally repressed by p53 (Chang et al., 2007). In fact, miR-34a has been shown to target and repress various genes that oppose p53 function, including Bcl2, Birc3, DcR3, Myb, E2F3, Cdk4, Cdk6, Cyclin D1 Cyclin E2 and Sirt1 (Chang et al., 2007; He et al., 2007; Navarro et al., 2009; Sun et al., 2008; Welch et al., 2007; Yamakuchi et al., 2008). Thus, just as p53 upregulates p21 to regulate downstream repression, p53 also induces miR-34a to downregulate a number of target genes.

Furthermore, p53 has recently been linked to the activation of several additional microRNAs (including miR-192, miR-145, miR-215, miR-107) that each has its own distinct subset of target genes. For example, p53 induces the transcription of miR-192 which serves to downregulate dihydrofolate reductase (a target of commonly used chemotherapeutics) (Song et al., 2008). miR-192, along with miR-215, also inhibit the expression of key cell cycle regulators including Cdc7 among others (Georges et al., 2008). miR-145 downregulates important cellular factors such as c-Myc (an oncogenic canonical p53-repression target), insulin receptor substrate-1 (IRS-1), Oct4, Sox2, and Klf4 (reprogramming factors) (Sachdeva et al., 2009; Shi et al., 2007; Xu et al., 2009), while miR-107 downregulates hypoxia inducible factor (Yamakuchi et al., 2010).

p53 has also been linked to the processing of microRNA, facilitating microRNA maturation without necessarily inducing transcriptional activation. p53 interacts with Drosha to facilitate cleavage of the stem-loop precursor to activate miR-16-1, miR-26a, miR-145, miR-143 and miR-206 (among others), which serve to downregulate genes

such as CDK6 and K-Ras (Suzuki et al., 2009). Either by transcriptional or non-transcriptional means, the positive regulation of microRNA expression by p53 adds another mechanism by which p53 inhibits both gene and protein expression of target genes.

Long non-coding RNAs

In addition to microRNAs and other small non-coding RNAs, thousands of long non-coding RNAs (lncRNA) are encoded by the genome. Recent studies have revealed that most of the eukaryotic genome is transcribed, but only a small proportion (5-10%) is stably expressed (mRNAs and spliced non-coding RNAs), with only ~1% of RNA transcripts coding for protein. Thus, myriad of non-protein coding RNAs are indeed expressed from the genome (Ponting et al., 2009). Long non-coding RNAs are defined as transcripts over 200 nucleotides in length that do not code for a protein sequence. This is to distinguish this species of RNA from the other classes of small RNAs (e.g. microRNAs).

Overall, the regulation and function of long non-coding RNAs is poorly understood as compared to other classes of RNA (both coding and non-coding), and much effort has gone into separating legitimate lncRNA from transcriptional “noise.” Despite the finding that lncRNAs sequences are not as evolutionarily constrained as other RNA classes, a variety of functions have been described for specific lncRNAs (Ponting et al., 2009). Of interest and relevance to p53’s role as a transcription factor, lncRNAs have been shown to contribute to epigenetic control, largely resulting in gene repression (Koziol and Rinn, 2010). Mechanistically, lncRNAs have been found to interact and

modulate chromatin modifying complexes (Khalil et al., 2009). The lncRNA, HOTAIR, for example, interacts with Polycomb repressive complexes (Rinn et al., 2007), and may represent only one of hundreds (if not thousands) of lncRNAs that share this function (Zhao et al., 2010). Thus, lncRNAs could represent another class of downstream mediators of p53-dependent repression.

Consistent with this idea, a recent study connected p53 to the world of long-intergenic non-coding RNAs (lincRNAs) (Huarte et al., 2010). lincRNAs represent a special class of evolutionary conserved lncRNA that are defined by having an open chromatin domain, and thus primary transcript, longer than 5 kb that does not overlap with any known protein coding gene or microRNA (Guttman et al., 2009). Huarte et al (2010) identified long intergenic RNA-p21 (lincRNA-p21), as a *bona fide* downstream target of p53 that serves to act as a key repressor through its interaction with heterogeneous ribonucleoprotein K (hnRNP-K) (Barsotti and Prives, 2010; Huarte et al., 2010).

To identify potential lincRNA targets of p53, the authors employed a custom tiling lincRNA microarray using two mouse cell lines engineered to induce p53 expression. Several lincRNAs that are potential targets of p53 were identified, and they focused on one of these, lincRNA-p21, named for its proximity to the p53-target gene p21. Importantly, interrogation of the human linc-RNA-p21 ortholog confirmed both sequence and regulatory conservation (Barsotti and Prives, 2010).

To ascertain the function of lincRNA-p21 within the p53 pathway, the authors induced p53 expression and knocked down lincRNA-p21. Using microarray analysis of protein coding genes, the authors found a large set of genes that were de-repressed in

response to lincRNA-p21 ablation. This set of genes was highly represented in the set of genes normally repressed by p53. This is a significant finding as it implicates lincRNA-p21 as a potent downstream mediator of p53-dependent transcriptional repression that acts on a very large scale (Barsotti and Prives, 2010).

As described above, p21 itself has also been implicated in repression of numerous p53 target genes. Nevertheless, lincRNA-p21 knockdown does not affect p21 mRNA or protein levels despite their close proximity. Furthermore, the set of genes regulated by lincRNA-p21 does not seem to overlap with the set of genes regulated by p21 previously identified by other studies, suggesting that lincRNA-p21 acts independently from p21 (Barsotti and Prives, 2010) and thus represents the founding member of a novel class of p53 targets that mediate downstream transcriptional repression.

p53 function

Lessons from mouse models

As previously stated, p53 facilitates various anti-proliferative cellular programs in response to stress. To do so, p53 activates and represses a large number of genes whose protein products regulate processes such as cell cycle arrest, quiescence, senescence, differentiation, and apoptosis. In addition to these fundamental processes, a role for p53 target genes have also been implicated in a wide variety of processes such as DNA repair, angiogenesis, metabolism, fertility, immunity, and even cell survival. The functions of such p53-target genes have indeed helped to decipher, and will continue to shed light on the precise mechanisms by which p53 suppresses tumor formation (see below).

Along with studies that elucidate the functions of specific p53 target genes, mouse models have greatly contributed to our understanding of p53's tumor suppressive capabilities. In this setting, the *in vivo* effects of p53 deletions, activation and mutation, can be assessed. As such, the contribution of the various p53 activities (e.g. apoptosis, cell cycle arrest, senescence) to tumor protection can be evaluated.

p53 null-mice are extremely tumor prone, succumbing to tumor formation with a median age of 5.5 months (Donehower et al., 1992). This first mouse model of p53 function clearly established the mouse as a viable system in which to study p53 tumor suppressive function and led to a plethora of *in vivo* experiments that have aimed to more precisely examine such functions (Reviewed in (Lozano, 2010)). Here, we will focus on experiments that have specifically examined p53-dependent apoptosis, cell cycle arrest and senescence.

As a potential means by which p53 may suppress tumor formation, initial studies aimed at characterizing the ability of p53 to initiate apoptosis (without addressing whether apoptosis specifically contributes to tumor suppression). To do so, these experiments compared the p53-null mice to wild type mice in response to various perturbations. For example, gamma-irradiation and etoposide cause cell death of thymocytes in wild-type, but not p53-null mice (Clarke et al., 1993; Lowe et al., 1993). Irradiating the developing embryo also leads to p53 dependent death in the central nervous system (Lee et al., 2001) and intestine (Clarke et al., 1994; Merritt et al., 1994).

Subsequent studies addressed the tumor suppressive function of p53-dependent apoptosis. For example, certain studies made use of mouse models which carry the deletion of a distinct tumor suppressor protein, Rb (mentioned above in the context of

indirect repression). In one experiment, inactivation of Rb initiates choroid plexus papillomas that develop slowly due to a concomitant increase in cell death (Symonds et al., 1994). Deletion of p53 prevents this cell death and increases the rate of tumorigenesis. Inactivation of Rb in a distinct cell type causes apoptosis and degeneration of the retina. p53 deletion again rescues this cell death and allows formation of tumors (Howes et al., 1994), clearly demonstrating a role for p53-dependent apoptosis in tumor prevention. Finally, a recent study generated a “knock-in” mouse that expresses a version of p53 lacking the proline rich region (Toledo et al., 2006). In opposition to the cell culture studies showing that this mutant is defective for apoptosis, this mutated version of p53 *in vivo* is unable to facilitate cell cycle arrest but does retain partial apoptotic function (Toledo and Wahl, 2006). Importantly, these mice do not spontaneously develop tumors (Toledo et al., 2006).

Despite these findings, additional *in vivo* experiments suggest that p53-mediated apoptosis is not solely responsible for the tumor suppressive capabilities of p53 (Vousden and Prives, 2009). PUMA is a p53-target gene necessary for apoptotic response to p53 activation in many tissues (Yu and Zhang, 2003). Despite being a key mediator of p53-dependent cell death, PUMA-null mice are not prone to developing spontaneous tumors (Michalak et al., 2008). This suggests that p53 can still suppress tumor formation even in the absence of robust cell death. However, in certain contexts such as Myc-driven tumors, loss of PUMA does indeed promote tumorigenesis (Garrison et al., 2008; Hemann et al., 2004). Further studies have addressed the contribution of DNA-damage induced p53-dependent apoptosis to tumor suppression. Surprisingly, these experiments suggest that p53-dependent apoptosis is not necessary for p53-dependent tumor

suppression in response to the DNA-damage caused by gamma-irradiation (Christophorou et al., 2005; Christophorou et al., 2006; Lozano, 2010).

Collectively, these findings suggest that while apoptosis does appear to be important, other mechanisms also contribute to p53's tumor suppressor activity. One of the most well-known functions of p53 is the inhibition of cellular proliferation and growth. Through transactivation of p21 and other target genes (discussed below), p53 blocks progression of the cell cycle, causing either transient arrest (el-Deiry et al., 1993; Harper et al., 1993) or permanent senescence (Noda et al., 1994). To test the hypothesis that cell cycle arrest/senescence contributes to tumor suppression, a mutant p53 knock-in mouse model was employed. In human tumors, residue R175 is a "hot-spot" for p53 mutations. As opposed to the R175H mutation, which completely disrupts p53 conformation (Sigal and Rotter, 2000), the R175P mutation selectively abolishes p53's ability to initiate apoptosis, but only partially affects p53-induced cell cycle arrest. In fact, the R175P retains the ability to transactivate p21, but is defective at binding and activating certain pro-apoptotic genes (Ludwig et al., 1996; Rowan et al., 1996). The equivalent mouse mutation falls at residue 172, and it was this 172P mutation that was knocked into mice. In perfect agreement with observations made with the human mutant, gamma-irradiation in mice homozygous for this mutation did not cause apoptosis in thymocytes or the developing central nervous system (just like in p53^{-/-} mice). As opposed to p53^{-/-} mouse embryonic fibroblasts (MEFs), MEFs generated from these mice did undergo partial cell cycle arrest. Importantly, the p53 R172P mice are reasonably well protected from spontaneous tumorigenesis, and develop tumors with a median of 11.5 months (Reviewed in (Vousden and Prives, 2009)). p53^{-/-} mice, as

previously stated, die due to tumor formation at a median age of 5.5 months. Thus, cell cycle arrest indeed contributes to tumor suppression by p53. Furthermore, the cell cycle arrest initiated by p53 R172P also afforded protection from genomic instability, as the lymphomas and sarcomas that did eventually develop in these mice were found to be diploid (Liu et al., 2004). The importance of p21 was also assessed in this context. Mice homozygous for p53 R172P that also lack p21 develop tumors on a similar time scale as p53^{-/-} mice, and the lymphomas/sarcomas isolated from such mice are aneuploid (Barboza et al., 2006). This suggests that p21 is a key mediator of tumor suppression by p53 (in the absence of apoptosis).

In addition to its role in cell cycle arrest, p21 also contributes p53-mediated senescence (permanent cell cycle arrest). The importance of senescence to tumor suppression has been revealed by several mouse models. For example, E μ -Myc induced lymphomas that are unable to undergo apoptosis due to an overexpression of Bcl-2 are able to respond to chemotherapy through the activation of p53/p16-dependent senescence (Schmitt et al., 2002). Furthermore, the induction of DNA-damage, by oncogene activation or telomere dysfunction, also activates p53-induced senescence in vivo (Cosme-Blanco et al., 2007; Deng et al., 2008; Feldser and Greider, 2007; Halazonetis et al., 2008).

Additional evidence of the importance of senescence to tumor suppression comes from mouse models of p53-reactivation. In HrasV12-driven carcinomas and sarcomas of p53^{-/-} mice, reactivation of p53 leads to tumor regression via senescence, but not apoptosis (Ventura et al., 2007; Xue et al., 2007). Although studies of cellular senescence in cell culture would suggest this cell fate would simply stabilize the disease

and not cause its regression, Xue et al reported activation of the immune response to facilitate clearance of senescent tumor cells (Vousden and Prives, 2009; Xue et al., 2007).

p53 target genes: the role of transcriptional activation and repression

Taken together, the mouse models described above have revealed that p53-induced apoptosis as well as p53-induced cell cycle arrest/senescence contribute to p53's tumor suppressive capabilities. p53 enacts such cellular programs predominantly through the activation or repression of a plethora of downstream target genes. The elucidation of target genes that function outside of these traditional categories have also provided insight into novel p53 functions.

Apoptosis

In response to p53 overexpression, or p53 activation by various stimuli, cell death occurs in a cell/tissue-type specific manner. While p53 can utilize non-transcriptional functions to augment apoptotic processes (a topic of intense research within the p53 field) (Palacios et al., 2008; Vaseva et al., 2009; Vaseva and Moll, 2009), it is clear that the transactivation of multiple p53 target genes greatly contribute to p53's ability to induce apoptosis (Haupt et al., 2003). These target genes may function in either the extrinsic or intrinsic apoptotic pathways. The extrinsic apoptosis pathway involves the binding of a pro-apoptotic ligand (e.g. Apo2L/TRAIL and CD95L/FasL) to a death receptor (e.g. DR4/DR5 and CD95/Fas) on the cell surface. This process triggers activation of the caspase cascade which cleave numerous cellular substrates that lead to many of the morphological features observed in apoptotic cells (reviewed in (Fulda and Debatin, 2006)). The intrinsic, or mitochondrial pathway, is a very complex pathway that involves

a shift in the balance of Bcl-2 family members toward pro-apoptotic members at the mitochondrial membrane. This shift in balance, along with other processes, causes permeabilization of the mitochondrial membrane, release of cytochrome C, formation of the apoptosome and subsequent caspase-mediated apoptosis (reviewed in (Debatin et al., 2002; Haupt et al., 2003; Kroemer, 2001)).

While the extrinsic apoptosis can occur independently of p53, p53 induces target genes that enhance the activity of this pathway (reviewed in (Zuckerman et al., 2009)). For example, p53 activates the transcription of both death receptors CD95/Fas and DR5/KILLER (TRAIL receptor 2) (Takimoto and El-Deiry, 2000), and both such receptors are induced by p53 in a cell-type and stimulus specific manner (Haupt et al., 2003). Additionally, p53 induces the expression of the pro-apoptotic ligands, TRAIL and FasL (Kuribayashi et al., 2008; Maecker et al., 2000). Finally, the transmembrane protein PERP is induced by p53 and has been shown to play a role in p53-mediated apoptosis (Attardi et al., 2000; Singaravelu et al., 2009).

p53 also transcriptionally activates key players in the intrinsic pathway and it is within this context that p53's function as a pro-apoptotic factor is most well-characterized. The Bcl-2 family proteins are key regulators of intrinsic apoptosis and are divided into three categories, pro-survival factors (e.g. Bcl-2 and Bcl-X_L), pro-apoptotic factors structurally similar to Bcl-2 (e.g. Bax and Bak) and pro-apoptotic BH3-only members (e.g. Puma, Noxa, Bid). One of the major roles of p53 within this pathway is the transcriptional upregulation of Bax and the BH3-only pro-apoptotic Bcl-2 family members, Puma, Noxa and Bid. Puma promotes oligomerization and mitochondrial translocation of Bax, ultimately causing apoptosis. Puma, which contains a higher

affinity p53-binding site than Bax (Kaesler and Iggo, 2002), potentially represents the key mediator of p53-dependent cell death as both cell culture and mouse studies have revealed a dependence on Puma expression for p53-mediated cell death (Jeffers et al., 2003; Yu et al., 2003; Yu and Zhang, 2003). Finally, p53 also transactivates several other pro-apoptotic proteins, such as PIDD, Apaf-1 and Caspase 6, which function in events downstream of mitochondrial permeabilization (reviewed in (Haupt et al., 2003; Yu and Zhang, 2005)).

Apoptosis: A role for transcriptional repression

p53-mediated transcriptional repression has also been implicated in the initiation/execution of cell death (reviewed in (Ho and Benchimol, 2003)). Despite the important findings discussed above, in certain situations transcriptional activation mediated by p53 is dispensable for its ability to cause apoptosis. For example, p53 can still facilitate cell death in the presence of transcription and translation inhibitors, suggesting that new mRNA and protein synthesis are unnecessary for this process (Caelles et al., 1994; Wagner et al., 1994). In line with these findings, transactivation deficient p53 mutants can still cause cell death in multiple cell lines (Ho and Benchimol, 2003), though the interpretation of such findings are difficult due to later studies which show specific remnant activity of such mutants (Baptiste-Okoh et al., 2008b). Furthermore, versions of p53 that are lacking the proline-rich region are unable to facilitate apoptosis in cell culture. Importantly, these p53 mutants retain their ability to transactivate target genes (including canonical targets such as p21, Mdm2 and Bax), but have lost their ability to repress transcription (Sakamuro et al., 1997; Venot et al., 1998;

Walker and Levine, 1996). Again, caution must be used in the interpretation of such findings, as transactivation was not completely diminished by this mutation (Venot et al., 1998). In an analogous experimental situation, the overexpression of cellular factors that inhibit p53's repression, but not transactivation, capabilities (e.g. Bcl-2, adenovirus E1B 19 K protein, or WT-1) correlate with a reduction in p53-dependent cell death (Maheswaran et al., 1995; Sabbatini et al., 1995; Shen and Shenk, 1994). Taken together, these results suggest that p53-dependent apoptosis can be separated from transcriptional activation and are in line with the hypothesis that transcriptional repression may play an important role in this process.

Consistent with this hypothesis, p53 down-regulates the transcription of a number of pro-survival factors. For example, p53 represses Bcl-2, Survivin, Map4, PIK3CA (the catalytic subunit of PI3K), and p202. Strikingly, the ectopic expression of all such gene products inhibits p53's ability to cause cell death (Chiou et al., 1994; D'Souza et al., 2001; Hoffman et al., 2002; Mirza et al., 2002; Murphy et al., 1996; Singh et al., 2002; Wang et al., 1993). Furthermore, antisense oligonucleotides to PI3KCA decreased cell survival (Singh et al., 2002). Additional studies using stress-specific conditions further confirm the importance of repression to p53-mediated apoptosis. For example, one study found that HCT116 colon cancer cell underwent apoptosis after treatment with the chemotherapeutic 5FU, and that under this condition p53 primarily acts as a transcriptional repressor, with 189 genes repressed and only 41 activated. Furthermore, RNAi-mediated inhibition of two of these repressed genes, PLK and PTTG, was sufficient to induce apoptosis (Kho et al., 2004). Similarly, p53-mediated apoptosis in response to hypoxia correlated with widespread gene repression, and not with the p53-

mediated activation of canonical target genes. In fact, such studies even revealed a preferential association of p53 with co-repressors rather than co-activators (Hammond et al., 2006; Koumenis et al., 2001). In addition, p53-mediated upregulation of certain microRNAs, such as miR-34a, are known to contribute to apoptosis (Raver-Shapira et al., 2007). microRNAs, including miR-34a, exert their function through the downregulation of mRNA expression or protein expression. For example, miR-34a targets and represses anti-apoptotic Bcl-2 (Hermeking, 2010). Collectively, these results suggest that in addition to activating pro-apoptotic genes, p53-mediated repression of pro-survival genes represents an important component of p53's ability to cause efficient apoptosis.

Cell cycle arrest/Senescence

Cell cycle checkpoints regulate key transitions of eukaryotic cell cycle progression (Hartwell and Weinert, 1989; Pietenpol and Stewart, 2002). Such checkpoints ensure the error-free completion of events that must occur prior to proceeding to the next cell cycle phase. In general, cells may temporarily arrest at checkpoints in order to allow for repair of cellular damage, abrogation of extracellular stress signals, or acquisition of growth factors (Pietenpol and Stewart, 2002). In contrast, cellular senescence represents an irreversible cell cycle arrest (Qian and Chen, 2010). Senescence is also triggered by cellular stressors, including telomere shortening (Harley et al., 1990), DNA damage (te Poele et al., 2002), and oncogene activation (Serrano et al., 1997). The p53 tumor suppressor protein is a key cellular factor involved in both cell cycle arrest and cellular senescence.

G1/S checkpoint: p53

S-phase represents the period in which eukaryotic cells replicate their genome. The G1/S cell cycle checkpoint prevents DNA replication in the situation where cells harbor damaged DNA, and it is within this context that p53 plays its most prominent role in cell cycle control (Reviewed in (Giono and Manfredi, 2006)). Both overexpression of ectopic p53 (Lin et al., 1992) or DNA-damage activated p53 cause a G1/S arrest ((Kastan et al., 1991). While multiple target genes of p53 are thought to contribute to G1/S arrest (BTG2, hCDC4b, GADD45) (Helton and Chen, 2007), the major mediator of p53's ability to inhibit G1/S progression arrest is the cyclin-dependent kinase inhibitor p21 (el-Deiry et al., 1994; el-Deiry et al., 1993). The main target for inhibition by p21 is cyclin-dependent kinase-2 (cdk2) (Brugarolas et al., 1998). When active, cdk2 phosphorylates and inactivates the pRb family members, thereby freeing and activating E2F-dependent transcription. After p53-mediated induction of p21, however, the growth suppressive form of Rb (hypophosphorylated) accumulates. Owing to its role as a key mediator of the p53-response, expression of p21 alone (in the absence of p53) is sufficient to cause growth arrest at the G1/S transition (and also the G2/M transition) (Rousseau et al., 1999). Furthermore, both p21-null mouse embryonic fibroblasts and HCT116 p21^{-/-} human colorectal cancer cells have an impaired G1/S arrest after DNA-damage (Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1995). Despite the central importance of p53 to G1 arrest, the initial delay of S-phase following DNA-damage occurs through p53-independent mechanisms. Such mechanisms (such as

ATM/Chk2 mediated inhibition of Cdc25A, degradation of Cyclin D1) are transcription-independent, and occur with fast kinetics. The p53 response extends this initial delay through transcriptional activation of target genes such as p21 and thus is important for the maintenance of the G1/S arrest (Giono and Manfredi, 2006).

G2/M Checkpoint: p53

The G2/M transition relies on activation of the Cyclin B1/Cdc2 complex, also known as the maturation promoting factor (MPF). This complex is activated by translocation to the nucleus and subsequent dephosphorylation of inhibitory phosphorylation sites by the Cdc25 phosphatases, and further activated by phosphorylation of distinct residues by the cdk-activating kinase (CAK) (Reviewed in (Giono and Manfredi, 2006)). These processes trigger entry into mitosis, the cell-cycle phase in which a cell must accurately segregate its genetic material into two daughter cells.

While many p53-independent mechanisms block entry into mitosis, the central importance of p53 to the maintenance of G2 arrest has been demonstrated under multiple conditions (Reviewed in (Taylor and Stark, 2001)). For example, expression of the viral proteins HPV-E6 and SV40 Large T antigen, both of which bind to and inactivate p53 function, allow irradiated IMR-90 cells to enter mitosis with either a higher frequency or faster kinetics (Chang et al., 1997; Thompson et al., 1997). However, other cellular targets of HPV-E6 or SV40 large T-antigen may also contribute to these phenotypes (Taylor and Stark, 2001).

A more definitive role for p53 was established by experiments conducted in the human colorectal cancer cell line HCT116 and HCT116 p53^{-/-} derivatives, in which p53 has been inactivated by homologous recombination (Bunz et al., 1998). Ionizing radiation initially arrested both HCT116 cells and HCT116 p53^{-/-} cells in G2, precluding entry into mitosis. In the absence of p53, however, this arrest was only transient. Wild-type p53-containing cells, in contrast, experienced prolonged G2 arrest. Furthermore, similar experiments were performed with HCT116 p21^{-/-} cells. These cells also arrested in G2 for only a short period of time and eventually entered mitosis (Bunz et al., 1998). Thus, p53 and its key target gene p21 are required for maintenance of a stable G2 arrest.

Initial G2-arrest, on the contrary, can be facilitated by p53-independent means. Two small molecules, caffeine and the protein kinase-inhibitor UCN-01, allow cells overcome this initial arrest and have been used to more precisely define the role of p53 at the G2/M transition. For example, MCF-7 cells which have been engineered to express HPV-E6 arrest in G2 in response to ionizing radiation. Yet, addition of either an analog of caffeine or UCN-01 allows abrogation of this arrest (Wang et al., 1996). Similarly, G2 arrest in p53^{-/-} MEFs is also diminished by caffeine (Powell et al., 1995). Importantly, in cells with wild-type p53 function, the G2 arrest can not be overcome by the addition of such small molecules. Thus, two distinct pathways work in conjunction to arrest cells in G2: p53-dependent pathways insensitive to caffeine/UCN-01, and p53-independent pathways that can be overcome in cells lacking p53 (Taylor and Stark, 2001).

Experiments utilizing DNA-damage caused by cellular stressors like ionizing radiation have clearly delineated a role for p53 at the G2/M transition. However, DNA-

damage causes the activation of multiple cellular pathways. In order to define a clearer role for p53 at this cell cycle checkpoint, p53 has been ectopically expressed or activated in the absence of cellular stress. In certain cellular systems (such as rat embryonic fibroblasts carrying a temperature sensitive version of p53) activation of p53 gives rise to G1 arrest, with very few cells arrested at the G2/M transition (Michalovitz et al., 1990). However, in different settings p53-expression does give rise to G2 arrest. For example, p53-null human fibroblasts that have been engineered to express tetracycline regulatable p53 were synchronized at early S-phase by the addition of mimosine. After removal of mimosine, induction of p53 gave rise to G2/M arrested cells with flattened interphase morphology (Agarwal et al., 1995; Taylor et al., 1999a; Taylor et al., 1999b). In addition, similar studies have confirmed p53's ability to arrest various cell types in G2. Thus, p53 can cause G2 arrest in the absence of DNA-damage, or prolong the G2 arrest in response to DNA-damage (Taylor and Stark, 2001).

p53 utilizes multiple mechanisms to regulate the G2/M transition, with most involving regulation of the CyclinB/Cdc2 complex. Acting as a transcription factor, p53 positively and negatively regulates a plethora of target genes that function at the G2/M transition, as well as during mitosis itself. Several targets of p53-mediated transactivation contribute to the G2 arrest, with p21 again taking center stage.

p21 induction in various cell types causes both G1 and G2 arrest. Cells with 4N DNA content following p21 induction do not contain condensed chromosomes, indicating that cells are indeed arrested in G2 and have not entered mitosis. Thus, p21 expression is sufficient for G2 arrest (Bates et al., 1998; Medema et al., 1998; Niculescu et al., 1998). Further, in some systems p21 expression is also necessary for G2 arrest.

For example, HCT116 p21^{-/-} cells fail to stably arrest in G2 after ionizing radiation (Bunz et al., 1998). This failure to arrest is accompanied by high levels of Cdc2 activity. Interestingly, cells that do aberrantly enter mitosis fail to complete such mitoses, containing multiple spindle poles which may have interfered with cytokinesis (Bunz et al., 1998).

Multiple mechanisms have been proposed for p21-mediated G2/M cell cycle arrest. First, p21 can inhibit Cdc2 activity through direct interaction with the Cyclin B1/Cdc2 complex (Taylor and Stark, 2001), though *in vitro* experiments have revealed that p21 inhibits Cdc2 less efficiently than other Cdk/Cyclin complexes (Harper et al., 1995). In an analogous situation, p21's ability to inhibit Cdk2 indirectly inhibits Cdc2 activity in *Xenopus* extracts (Guadagno and Newport, 1996). p21 may also block activation of Cdc2 by CAK (Smits et al., 2000), though the mechanism for this process is unclear (Taylor and Stark, 2001). Another proposed mechanism relies on the interaction of p21 and PCNA, a processivity factor for DNA polymerases delta and epsilon required for DNA synthesis as well as repair (Waga et al., 1994). Such experiments demonstrated that a p21 mutant incapable of binding Cdks that retains the ability to bind to PCNA facilitated G2 arrest in DLD1 colorectal cancer cells (Cayrol et al., 1998). Since Cdc25C has also been observed to interact with PCNA, one study has put forward the hypothesis that p21-PCNA interaction disrupts the ability of Cdc25C to bind the CyclinB1/Cdc2 complex (Ando et al., 2001). In line with these observations, another study demonstrated that p21 does physically separate Cyclin B1/Cdc2 complexes from Cdc25C, thus preventing activation of the complex (Charrier-Savournin et al., 2004). In addition, p21-

mediated transcriptional repression may contribute to p21's ability to maintain a stable G2 arrest (see below).

Aside from p21, p53 also induces several other target genes that participate in the G2/M checkpoint. Gadd45 represents one such p53-inducible gene that functions in the G2 arrest. Whereas normal mouse lymphocytes arrest in G2 in response to ultraviolet radiation (UV), Gadd45-null mouse lymphocytes are unaffected by UV treatment (Wang et al., 1999a). However, the G2 arrest response after irradiation was intact in such cells, suggesting that the action of Gadd45 is stimulus specific (Wang et al., 1999a). Gadd45 has been proposed to facilitate G2 arrest through its ability to dissociate the CyclinB1/Cdc2 complex (Jin et al., 2000; Zhan et al., 1999).

14-3-3 sigma is another p53-inducible gene that also plays a role in the G2/M checkpoint. 14-3-3 sigma is induced in response to ionizing radiation, and its overexpression results in G2 arrest. In contrast to p21 and Gadd45, 14-3-3 sigma acts by preventing the nuclear translocation, and thus inhibiting the activity, of Cdc2 (Hermeking et al., 1997; Taylor and Stark, 2001). Finally, p53 also induces various additional target genes believed to contribute to G2 arrest, including BTG2, B99, HZF, MCG10, and Reprimo (Helton and Chen, 2007).

Senescence: p53

Cellular senescence was first observed in the context of normal human diploid fibroblasts (Reviewed in (Qian and Chen, 2010)). Such fibroblasts undergo a limited number of passages before entering a non-proliferative, but metabolically active state, which is defined as replicative senescence (Hayflick, 1965; Hayflick and Moorhead,

1961). Morphologically, senescent cells are enlarged and flattened. They are characterized by an inability to synthesize DNA and expression of senescence-associated-Beta-galactosidase (Dimri et al., 1995).

In senescent cells, p53 levels and activity are increased. Importantly, overexpression of p53 is sufficient to induce senescence in p53-null cells. Furthermore, inhibition of p53 by viral proteins such as HPV-E6 or SV40 Large T-antigen extend the lifespan of a cellular population. Interference with p53 activity through loss of heterozygosity or expression of a dominant negative p53 also causes a delay in senescence. Taken together, such results delineate an important role for p53 as a mediator of cellular senescence. In human cells, however, the inhibition of an additional factor (p16) is also required to bypass senescence. Similarly, DNA-damage induced senescence is greatly diminished in the absence of p53, but not abolished. These results suggest that p53-independent pathways also contribute to the senescent phenotype (Reviewed in(Qian and Chen, 2010)).

As mentioned above, several cellular stressors such as telomere shortening, DNA-damage and oncogene-activation can trigger the onset of senescence (Reviewed in (Qian and Chen, 2010)). Telomeres (which cap the end of chromosomes) are progressively shortened by each cellular population doubling, and it is the length of such telomeres that determines the cellular lifespan (Harley et al., 1990; Lundblad and Szostak, 1989). Uncapping of telomeres (removal of specific protein complexes from chromosome ends due to telomere attrition) causes a DNA-damage signal that leads to cellular senescence (d'Adda di Fagagna et al., 2003). Aberrant oncogenic activities also result in senescence, termed oncogene-induced senescence (OIS). For example, cells that are engineered to

express constitutively active Ras (RasV12) undergo premature senescence, a cellular state indistinguishable from replicative senescence (Serrano et al., 1997). In contrast, RasV12 expression combined with overexpression of other oncogenes (e.g. c-Myc, E1A, SV40 Large T-antigen) or loss of tumor suppressors like p53 or p16, causes cellular transformation (Chintala et al., 1997; Kemp et al., 1993; Land et al., 1983; Ruley, 1983). Thus, it is likely that p53 safeguards against aberrant mitogenic signaling by Ras by contributing to OIS. Ras-induced senescence depends on some combination of the Raf-Mek-Erk MAPK pathway, the JNK-p38 MAPK pathway, and alteration of intracellular reactive oxygen species (Reviewed in (Qian and Chen, 2010)). Ras-induced senescence is also accompanied by detectable DNA-damage foci. In fact, inactivation of the DNA-damage response pathway abrogates OIS and promotes cellular transformation, suggesting that DNA-damage resulting from uncontrolled Ras signaling greatly contributes to OIS (Di Micco et al., 2006). Thus, DNA-damage represents a component of both telomere-shortening induced senescence as well as OIS. Consistently, certain DNA-damaging agents, such as chemotherapeutic drugs, radiation and oxidative stress, induce premature senescence. This process is termed stress-induced senescence, or SIS.

p53 is activated and is a downstream effector of all three cellular senescence responses: replicative senescence, OIS and SIS (Reviewed in (Qian and Chen, 2010)). In replicative senescence, both ATM/Chk2 and ATR/Chk1 phosphorylate p53, stabilizing p53 levels and activating p53-dependent transcription. In OIS, p53 is activated by two major signaling pathways. The first involves the activation of the tumor suppressor ARF via the MAPK pathway. ARF subsequently interacts with Mdm2 and prevents Mdm2-mediated degradation of p53 (Zhang and Xiong, 2001). In the second, the DNA-damage

pathway responds to replicative stress caused by hyper-DNA replication associated with constitutive Ras signaling (Bartkova et al., 2006; Di Micco et al., 2006). As before, ATM/Chk2 and ATR/Chk1 activate p53 through phosphorylation and knockdown of such factors attenuates OIS (Mallette and Ferbeyre, 2007; Mallette et al., 2007)(Halazonetis et al., 2008; Toledo et al., 2008). Finally, both normal and tumor cells undergo SIS. In response to treatments such as doxorubicin, camptothecin, and cisplatin, the ATM/ATR-p53 axis is activated (Chang et al., 1999; Schmitt, 2007). However, as certain cancer cells devoid of wild-type p53 still undergo SIS, other factors aside from p53 must also play a role in inducing SIS in cancer cells (Chang et al., 1999; Qian et al., 2008).

Several p53-inducible genes contribute to cellular senescence. For example: p21, PML and PAI-I have all been shown to be sufficient for mediating senescence. p21, a crucial p53-mediator of the G1 and G2 cell cycle arrest, also plays a role in the induction of senescence. It is overexpressed in senescent cells (Noda et al., 1994), and induces senescence in p53-null H1299 cells (Wang et al., 1999b). Inactivation of p21 also leads to increased cellular lifespan. However, cells without p21 eventually enter senescence, and lack of p21 does not completely abolish SIS in tumor cells (Brown et al., 1997). Thus, p21 is sufficient, but not necessary for p53-mediated senescence. PML, another direct p53-target gene that can induce premature senescence, stabilizes p53 by promoting post-translational modifications of p53 and inhibiting Mdm2. PAI-I is considered to be a marker of cellular senescence, as it is upregulated in aging fibroblasts both *in vitro* and *in vivo*. PAI-I halts cell cycle proliferation by directly inhibiting uPA (a secreted protease that promotes the G1/S transition). Knockdown of PAI-I by siRNA causes an escape

from replicative senescence, suggesting that PAI-I is a critical modulator of senescence. Finally, the p53-mediated repression of target genes also contributes to senescence (see below). Thus, through regulation of transcription, p53 mediates cellular responses that lead to senescence in response to a variety of stimuli. However, as p53-mediated cell cycle arrest is often the first step in cellular senescence, the molecular mechanisms by which cells commit to permanent cell cycle arrest still awaits further exploration (Reviewed in (Qian and Chen, 2010)).

Cell cycle arrest and senescence: The role of p53-mediated repression

As described above, the ability of p53 to transactivate specific target genes contributes to the execution of cell cycle arrest and senescence. In addition, p53-mediated repression also plays a role in p53's ability to inhibit cell cycle progression. For example, p53-mediated G1 arrest not only relies on transactivation of p21, but also on repression of c-Myc, which occurs in a p53-dependent manner in multiple human and mouse cell lines. Ectopic expression of c-Myc interferes with p53-dependent G1 arrest, and instead enhances p53-mediated apoptosis (Ho et al., 2005). Thus, downregulation of c-Myc can be viewed as one mechanism by which p53 selects cellular outcomes in response to a given stress.

Transcriptional repression mediated by p53 also significantly contributes to inhibition of the G2/M transition. p53 is known to inhibit the expression of several key cellular factors that control this transition. As noted above, p53-dependent mechanisms generally converge on the activity CyclinB1/Cdc2 complex in order to maintain stable G2 arrest. Consistently, p53-mediated repression also targets the expression of both Cyclin

B1 and Cdc2. p53 overexpression in normal human fibroblasts caused inhibition of Cdc2 activity which was attributable in part to reduced levels of Cyclin B1 (Taylor et al., 1999b). Loss of Cyclin B1 expression occurred prior to G2 arrest due to transcriptional repression by p53 (Taylor et al., 1999b). Repression of Cyclin B was also observed in SKOV3 ovarian cancer cells in response to p53 activation. Such repression correlated with G2 arrest, and ectopic Cyclin B1 expression alone could overcome such arrest (Innocente et al., 1999). This suggests that repression of Cyclin B1 by p53 represents a crucial cog in the arrest machinery. p53 also transcriptionally represses Cdc2 expression, though this loss is observed after Cyclin B1 reduction. p53-dependent transcriptional repression of Cdc2 has been observed after ionizing radiation and ectopic p53 expression (Azzam et al., 1997; de Toledo et al., 1998; Passalaris et al., 1999; Taylor et al., 1999b).

The Cdc25C gene represents another key target of p53-mediated repression involved in blocking the G2/M transition (St Clair et al., 2004; St Clair and Manfredi, 2006). Cdc25C is a phosphatase that activates the Cyclin B1/Cdc2 complex (Morgan, 1997) and its overexpression inactivates the G2 checkpoint (without affecting the G1 checkpoint) (St Clair et al., 2004). Repression of this target therefore provides yet another mechanism for p53 to target the activity of the MPF. Other repression targets that function in G2 or M phases have been identified by individual studies, including but not limited to Cyclin B2 (Imbriano et al., 2005; Krause et al., 2000), Topoisomerase II alpha (Wang et al., 1997) and hMps1 (a mitotic kinase) (Bhonde et al., 2006).

A recent study has identified additional p53-repressed genes that function in the cell cycle (Spurgers et al., 2006). In fact, adenoviral-expressed p53 appears to downregulate more genes involved in the cell cycle than in any other cellular process, as

41% of all repressed genes fell under this category (Spurgers et al., 2006). A subset of these genes was also repressed in a p53-dependent manner in response to DNA-damage. For example, etoposide-activated p53 was found to repress the following cell cycle genes: Cyclin A2, Cdc25A, Pre1, Cdc6, Cdc20, Kif23, Kntc2, Nek2, Fen1, Tpx2, Chk1, Rrn2 and MCM6. Importantly, many of the genes identified by this study were repressed prior to evidence of cell cycle arrest, and thus are not downregulated as a secondary consequence of their expression being modulated by the cell cycle (Spurgers et al., 2006). This systematic study has thus revealed that p53-mediated repression plays a major role in cell cycle arrest. In addition, further analysis suggests that p53 also represses many genes involved in DNA repair, nucleotide metabolism, and DNA packaging (Spurgers et al., 2006).

p53-mediated repression has also been implicated in cellular senescence. For example, p53 may enforce the replicative lifespan of human cells through the repression of the human telomerase reverse transcriptase (which functions to lengthen telomeres) (Kanaya et al., 2000; Xu et al., 2000). Furthermore, p53 indirectly represses genes such as ID1 through the upregulation of Dec1, a factor sufficient for causing premature senescence (Qian et al., 2008). Finally, p53-dependent activation of the Rb family causes cell cycle exit from G2 and cellular senescence; such phenotypes are mediated by Rb-family-dependent transcriptional repression (Jackson et al., 2005; Lehmann et al., 2008).

The p53-dependent induction of microRNAs also has a significant role in cell cycle arrest and senescence. miR-34a contributes to both arrest and senescence through suppression of multiple cell cycle genes, including cyclin D1, Cyclin E2, Cdk4, Cdk6, Met, c-Myc, n-Myc, Cdc25C, Myb and Notch1 (Hermeking, 2010). Strikingly, antisense

RNA to miR-34a delays the onset of replicative senescence (Fujita et al., 2009). miR-192 targets dihydrofolate reductase and causes G1 and G2 arrest (Song et al., 2008). miR-145, on the other hand, targets c-Myc (Sachdeva et al., 2009). Taken together, p53-mediated repression (whether direct or indirect) clearly has an important role in the regulation of cell cycle arrest and senescence.

Stem cells and cancer stem cells: p53

In addition to the well studied roles for p53 in apoptosis and cell cycle arrest/senescence (described above), recent studies suggest that p53 regulates facets of stem cell biology, and that such regulation may prove to be an important tumor suppressive mechanism.

A major recent breakthrough in the stem cell biology field was the observation that the overexpression of four transcription factors (Oct2, Sox2, Klf4, and c-Myc) de-differentiates somatic cells to a state of pluripotency (Takahashi and Yamanaka, 2006). In general, however, the generation of such induced pluripotent stem cells (iPS) cells is quite inefficient. Strikingly, inhibition of p53 causes a dramatic increase in reprogramming efficiency. Though still unclear, the reprogramming process is thought to activate p53 through oncogenic stress as well as genomic aberrations (Reviewed in (Menendez et al., 2010; Tapia and Scholer, 2010)).

While depletion or mutation of p53 does increase the efficiency of iPS cell generation, such iPS cells acquire features related to tumorigenesis, such as impaired differentiation. In fact, a role for p53 in differentiation has previously been established. For example, p53-null mice develop polydactyly, and have defects in neural tube closure

and bone development. Furthermore, p53 has been shown to contribute to differentiation in multiple settings and plays a key role in establishing cell fate (e.g. mesodermal versus ectodermal tissue development, control of the differentiation programs of both skeletal muscle and mesenchymal progenitor cells) (Reviewed in (Menendez et al., 2010; Tapia and Scholer, 2010)).

Taken together, the findings described above suggest that the undifferentiated status of certain tumors may arise from p53 mutation (potentially in an adult stem cell). A recent study provided evidence for this hypothesis, showing that mutations of p53 correlate with breast tumor cells that display stem-cell like transcriptional patterns. Thus, loss of p53 function may generate a stem-cell like state that is further selected for during tumor progression (Mizuno et al., 2010).

As described above, p53 carries out multiple functions which may prevent reprogramming, or promote differentiation. Owing to inherent oncogenic stress and genomic aberrations associated with the iPS reprogramming process, p53's ability to cause apoptosis or senescence limits the capacity of a cell population to be reprogrammed. Other more specific methods by which p53 promotes differentiation have also been established, some of which rely on p53-mediated repression. For example, DNA damage causes the p53-dependent repression of Nanog (a gene required for self-renewal of embryonic stem cell (ESC)) with subsequent differentiation of ESCs. In effect, this prevents the unlimited renewal capacity of cells with DNA-damage (Lin et al., 2005). Furthermore, p53 induces miR-145 (Sachdeva et al., 2009) which has been shown to inhibit ESC self-renewal through downregulation of Oct4, Sox2, and Klf4 (Xu et al., 2009). Finally, induction of miR-34a also inhibits self-renewal and survival of

cancer stem cells through targeting factors such as Notch, HMGA2 and Bcl-2 (Ji et al., 2008).

Other tumor associated phenotypes: p53

p53 also plays additional anti-tumorigenic roles that are not associated with cell proliferation *per se*. For example, mutation of p53 leads to cell migration and tumor invasiveness. However, depletion of p53 from mice does not give rise to tumors that commonly metastasize, suggesting that the inhibition of migration and invasion is not a function of wild-type p53 (Muller et al., 2011). However, wild-type p53 does inhibit angiogenesis (the process by which new blood vessels are formed). Tumors initiate angiogenesis in order to maintain a supply of oxygen and nutrients necessary for survival. At least three mechanisms have been described for p53-mediated inhibition of angiogenesis: (1.) interference with regulators of hypoxia, (2.) inhibiting production of angiogenic factors, and (3.) up-regulating anti-angiogenic factors. Notably, p53-mediated transcriptional repression of the pro-angiogenic factors VEGF, bFGF, bFGF-BP (secreted factors) and Cox2 contribute to p53's ability to prevent new blood vessel formation. In fact, under conditions of severe hypoxia, p53 functions more as a transcriptional repressor rather than activator (Reviewed in (Teodoro et al., 2007)). Finally, p53 plays roles in the regulation of reactive oxygen species (ROS) and metabolism that also contribute to p53's ability to suppress tumor formation. Here, transcriptional repression of genes such as GLUT1, GLUT4 serve to inhibit glycolysis and oppose the Warburg effect (Reviewed in (Gottlieb and Vousden, 2010; Levine and Puzio-Kuter, 2010)).

FoxM1

As described above, several functions of p53 including cell cycle arrest and senescence, contribute to p53's ability to suppress tumor formation. Notably, p53 represses a large number of genes that function in the cell cycle. As such, we hypothesized that p53 may inhibit the expression of a master regulator of proliferation. One such master regulator is the forkhead transcription factor FoxM1.

FoxM1 is one member of a large class of transcription factors that share the evolutionarily conserved Winged Helix/Forkhead DNA binding domain. The Forkhead Box (Fox) transcription factor family, containing 55 distinct mammalian factors, is named for the two head-like structures in fork head *Drosophila* mutants that have developmental defects. Fox proteins are linked to several human congenital disorders and play important roles in human cancer (Reviewed in (Kalin et al., 2011)).

The expression of FoxM1 correlates with cells that are proliferating. FoxM1 is expressed in embryonic tissues as well as proliferating cells of mesenchymal and epithelial origin of both embryonic and adult tissue. Recent studies have carefully dissected the FoxM1 expression pattern in the developing mouse embryo, showing expression of FoxM1 in many cell and tissue types. (e.g. hepatoblasts, cardiomyocytes, smooth muscle cells, endothelial cells, thymocytes, developing skin, kidney and cartilage, etc.) In adults, however, FoxM1 expression is restricted to those tissues that have a high proliferation rate (e.g. testis, thymus, lung and intestine). Furthermore, FoxM1 is expressed in immature cell populations and stem cell germinal centers, but is

downregulated as cells differentiate (Reviewed in (Kalin et al., 2011; Laoukili et al., 2007)).

FoxM1 expression can also be reactivated in adults. For example, FoxM1 expression is reactivated after organ injury in order to facilitate regeneration (e.g after partial hepatectomy, pancreatectomy or lung injury), as well as during cancer formation. In line with this observation, FoxM1 is also expressed in a wide variety of cell lines and tumor-derived cell lines. In such cultured cells, FoxM1 levels are serum-inducible. Collectively, these observations demonstrate that FoxM1 is a proliferation-specific gene, inhibited in terminally differentiated or quiescent cells, but activated upon re-entry into the cell cycle (Reviewed in (Laoukili et al., 2007)).

In addition to being restricted to proliferating cells, FoxM1 expression fluctuates as function of the cell cycle. Both FoxM1 mRNA and protein levels begin to rise at the onset of S-phase, and continue through G2 and M phases, reaching maximum levels during G2 (Reviewed in (Laoukili et al., 2007)). FoxM1 protein is regulated in a Cdh1-dependent manner, degraded upon exit from mitosis/early G1 phase (Park et al., 2008)(Laoukili et al., 2008). Furthermore, FoxM1 transcriptional activity is also regulated by phosphorylation in a cell-cycle dependent manner. FoxM1 interacts with Cdk-Cyclin complexes, Rb, Cdc25B, and p19ARF, all of which regulate the transcriptional activity of FoxM1 (Reviewed in (Laoukili et al., 2007)).

The importance of FoxM1 to development is underscored by the finding that deletion of FoxM1 in mice is embryonic lethal due to defects in organogenesis, specifically within the heart, liver, lungs and pancreas (Reviewed in (Laoukili et al., 2007)). Therefore, FoxM1 expression is crucial for cellular proliferation in numerous

tissue types. These studies also demonstrated a role for FoxM1 in mitosis, as cardiomyocytes, hepatocytes and MEFs of FoxM1 null mice show evidence of polyploidy (Reviewed in (Laoukili et al., 2007)).

Consistent with the precise regulation of FoxM1 expression and activity by cell cycle associated factors, FoxM1 functions as a crucial regulator of the cell cycle. FoxM1 has been implicated in control of the G1/S transition as well as the G2/M transition. FoxM1 induces the expression of many genes that promote passage from G1 to S-phase as well as DNA-replication events, including but not limited to c-Myc, Cyclin A2, Cyclin D1/D2, Jnk1, Atf2, and Cdc25A (Laoukili et al., 2007; Wierstra and Alves, 2007a, b). Furthermore, FoxM1 activates members of SCF ubiquitin ligase complex, Skp2 and Cks1, which is required for the degradation of Cdk inhibitors p21 and p27 during G1 phase. Through induction of these factors, FoxM1 indirectly downregulates cell-cycle inhibitory factors and allows progression into S-phase (Reviewed in (Laoukili et al., 2007) and (Wierstra and Alves, 2007a)).

In addition to its role at the G1/S transition, FoxM1 is also a critical regulator of the G2/M transition and mitotic events (Reviewed in (Laoukili et al., 2007)). FoxM1 directly activates the transcription of the key cellular factors Cdc2, Cyclin B1, Cyclin B2, as well as Cdc25B and Cdc25C (phosphatases necessary for activation of the Cdc2/Cyclin B1 complex). Furthermore, FoxM1 also upregulates transcription of G2 and mitosis specific genes that ensure proper chromatin segregation and cytokinesis, including components of the APC (Cdc20), mitotic kinases (Plk-1, Nek2, AurkB), and mitotic spindle associated proteins/motor proteins (Survivin, Stathmin, CenpA/B/F). Consistent with its role in cellular proliferation, FoxM1-null MEFs and FoxM1-null

pancreata display premature senescence. Knockdown of FoxM1 in cell culture also results in cell cycle defects and senescence (Reviewed in (Laoukili et al., 2007)). More specifically, FoxM1 ablation has been demonstrated to impair M-phase entry and execution of mitotic events (Laoukili et al., 2005). The prolonged G2 arrest experienced by FoxM1 deficient cells can be overcome by ectopic overexpression of Cyclin B1, suggesting that this FoxM1-inducible gene is critical for mitotic entry (Laoukili et al., 2005). Furthermore, the fraction of FoxM1-ablated cells that eventually enter mitosis undergo various mitotic abnormalities (mitotic spindle defects, chromosome missegregation) and centrosome amplification that lead to mitotic catastrophe (Wonsey and Follettie, 2005) or endoreduplication leading to polyploidy and even aneuploidy (observed in FoxM1-null MEFs) (Wang et al., 2005).

FoxM1 is one of the most commonly overexpressed genes in human solid tumors (Pilarsky et al., 2004). FoxM1 was found to be overexpressed in a wide variety of tumors, including non-small cell lung cancers, head and neck squamous carcinomas, hepatocellular carcinomas, prostate adenocarcinomas, basal cell carcinomas, intrahepatic cholangiocarcinomas, colon carcinomas, infiltrating ductal breast carcinomas, anaplastic astrocytomas, glioblastomas, pancreatic carcinomas, gastric cancer, and acute myeloid leukemia (Reviewed in (Kalin et al., 2011)). Numerous mouse models have also addressed the role of FoxM1 in carcinogenesis. Overexpression of FoxM1 leads to increased tumor number and size after induction of tumors with various agents such as the chemicals found in tobacco smoke or BHT (lung cancer), as well as azoxymethane and dextran sodium sulfate (colorectal cancer). Furthermore, FoxM1 overexpression cooperates with SV40 large-T antigen expression to accelerate initiation and progression

of prostate adenocarcinomas. On the other hand, depletion of FoxM1 from cancer models decreases lung-tumor formation and delays the growth of hepatocellular carcinoma. More targeted studies have already begun to address the precise role of FoxM1 in various cell types in the process of tumorigenesis (Reviewed in (Kalin et al., 2011)).

FoxM1 has recently been linked to aspects of tumorigenesis not connected with cellular proliferation. For example, FoxM1 stimulates cell migration and invasion of cultured cells through the transcriptional activation of JNK1 and the matrix metalloproteinases MMP-2 and MMP-9. Furthermore, FoxM1 has been shown to initiate angiogenesis in pancreatic adenocarcinomas through direct transactivation of factors such as VEGF and Cox2 (Reviewed in (Kalin et al., 2011)). Finally, FoxM1 has been shown to contribute to stem cell biology and tumor initiating cells. FoxM1 expression is necessary for the maintenance of pluripotency of embryonal carcinoma P19 cells, as FoxM1 knockdown causes spontaneous differentiation into mesodermal derivatives, such as muscle and adipose tissue. The maintenance of the pluripotent state was found to be due to FoxM1 activation of Oct4, Sox2 and Nanog (Xie et al., 2010). Intriguingly, the results of another study also suggest a role for FoxM1 in the maintenance of a stem cell state. FoxM1 overexpression in primary human keratinocytes with stem/progenitor cell properties, but not differentiated keratinocytes, caused clonal expansion. Such FoxM1 overexpression also perturbed differentiation of keratinocyte progenitors and resulted in a phenotype similar to human epithelial hyperplasia. As FoxM1 overexpression leads to expansion of the progenitor cell population and deregulates differentiation, these results

suggest that FoxM1 overexpression may be a very early event in tumorigenesis (Gemenetzidis et al., 2010).

Given the opposing functions of FoxM1 and p53 and striking overlap of target genes (pro- versus anti-proliferative, pro- versus anti-angiogenic), we explored the hypothesis that p53 downregulates the transcription of FoxM1. Figure 1 displays select FoxM1-inducible genes and the cellular process that their protein products regulate. Highlighted genes denote the genes that have also been reported to be down-regulated by p53 (some of which are well-established targets, while others have been identified only by genome-wide expression arrays). Chapter 2 of this dissertation describes the phenomenon, mechanism and function of p53-mediated repression of FoxM1.

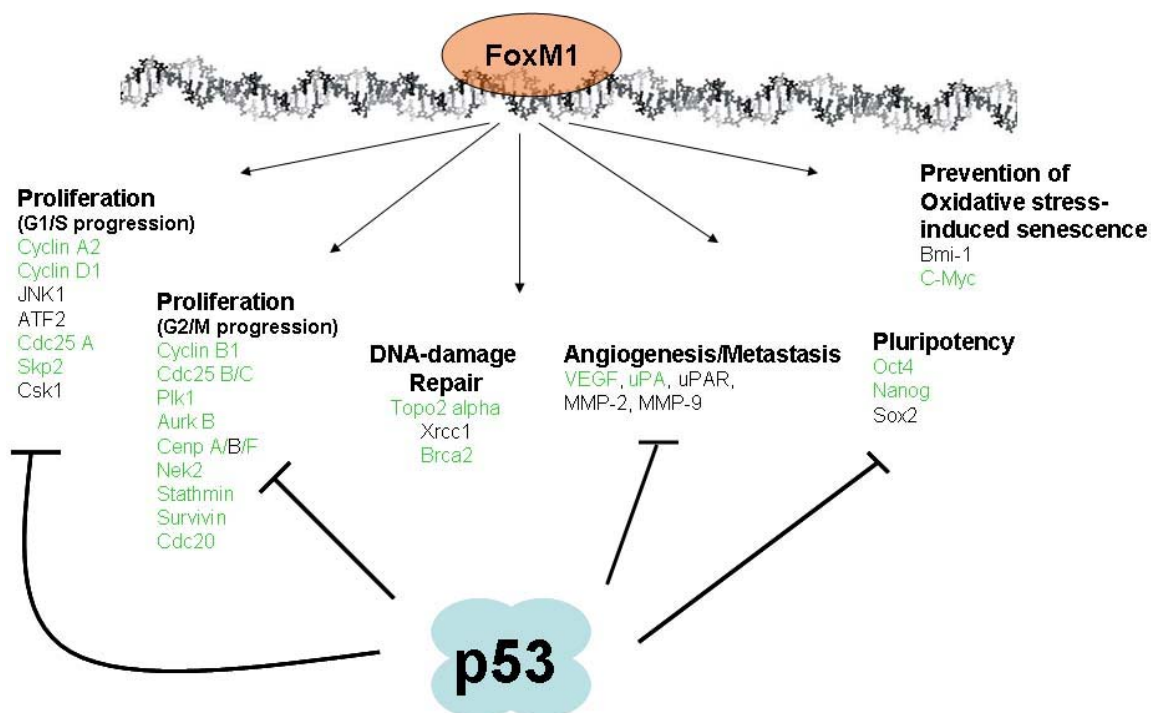


Figure 1

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Chapter 2



ORIGINAL ARTICLE

Pro-proliferative FoxM1 is a target of p53-mediated repression

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The p53 tumor suppressor protein acts as a transcription factor to modulate cellular responses to a wide variety of stresses. In this study we show that p53 is required for the downregulation of FoxM1, an essential transcription factor that regulates many G2/M-specific genes and is overexpressed in a multitude of solid tumors. After DNA damage, p53 facilitates the repression of FoxM1 mRNA, which is accompanied by a decrease in FoxM1 protein levels. In cells with reduced p53 expression, FoxM1 is upregulated after DNA damage. Nutlin, a small-molecule activator of p53, suppresses FoxM1 levels in two cell lines in which DNA damage facilitates only mild repression. Mechanistically, p53-mediated inhibition of FoxM1 is partially p21 and retinoblastoma (Rb) family dependent, although in some cases p21-independent repression of FoxM1 was also observed. The importance of FoxM1 to cell fate was indicated by the observation that G2/M arrest follows FoxM1 ablation. Finally, our results indicate a potential contribution of p53-mediated repression of FoxM1 for maintenance of a stable G2 arrest.

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Keywords: p53; FoxM1; transcriptional repression; p21; Rb; G2 arrest

Introduction

p53, a tumor suppressor protein that is frequently mutated in human primary tumors (Petitjean *et al.*, 2007), directs such processes as apoptosis, cell cycle arrest and senescence through transcriptional regulation of genes that control cell growth and survival (Das *et al.*, 2008; Riley *et al.*, 2008). Although a majority of p53 studies focus on transactivation, multiple gene array studies have shown that p53 also represses the transcription of a substantial number of genes (Wang *et al.*, 2001; Mirza *et al.*, 2003; Robinson *et al.*, 2003; Sax *et al.*, 2003; Kho *et al.*, 2004; Sun, 2006). As binding of p53 to its canonical response element is not always necessary for repression (Gridasova and Henry, 2005; Imbriano *et al.*, 2005), p53 makes use of several mechanisms to

impair mRNA expression from specific promoters (reviewed in Ho and Benchimol (2003) and Laptenko and Prives (2006)). Mechanisms that do not involve direct interactions of p53 with the repressed promoter often rely on activation of the cyclin-dependent kinase inhibitor p21/WAF1 (Spitkovsky *et al.*, 1997; Gottifredi *et al.*, 2001; Lohr *et al.*, 2003).

Although several reports have linked p53-mediated repression to p53-induced apoptosis (reviewed in Ho and Benchimol, 2003), many key cell cycle regulators are also repressed in a p53-dependent manner (Spurgers *et al.*, 2006). In particular, p53-mediated repression of c-myc was shown to be necessary for G1 arrest (Ho *et al.*, 2005). Many reports also document the ability of p53 to repress important G2/M regulators such as cyclin B (Krause *et al.*, 2000; Innocente and Lee, 2005), Plk1 (Jackson *et al.*, 2005; Incassati *et al.*, 2006), Cdc2 (Yun *et al.*, 1999; Taylor *et al.*, 2001), Cdc25C (Krause *et al.*, 2001; St Clair *et al.*, 2004; St Clair and Manfredi, 2006) and survivin (Hoffman *et al.*, 2002; Mirza *et al.*, 2002; Raj *et al.*, 2008). Although p53-independent mechanisms can block entry into mitosis after DNA damage, a role for p53 has been established at the G2/M transition (reviewed in Taylor and Stark, 2001; Giono and Manfredi, 2006). Specifically, p53 and p21 are crucial for the maintenance of a stable G2 arrest and for prevention of aberrant mitotic entry after DNA damage (Bunz *et al.*, 1998).

To further analyse the ability of p53 to regulate the cell cycle, and in particular the G2/M transition, we sought to identify targets of p53 that have critical roles in cell cycle progression. Although studies of global gene regulation by p53 have identified an abundance of p53-repression targets, relatively few repression targets have been the subject of careful analysis. Tremendous insight into the full p53 tumor suppressor arsenal can be gained by studying the mechanism and function of p53-mediated repression of important target genes.

Using microarray analysis, two studies independently identified FoxM1 (Trident, MPP-2) as a gene the mRNA of which is significantly downregulated upon p53 expression/activation (Bhonde *et al.*, 2006; Spurgers *et al.*, 2006). FoxM1, a forkhead-family transcriptional activator, is a major regulator of both the G2/M transition and mitotic progression, and is required for proper cell proliferation (Laoukili *et al.*, 2007). Its importance is underscored by the fact that FoxM1^{−/−} mice die *in utero* at day E18.5 (Krupczak-Hollis *et al.*, 2004). Not only is FoxM1 expression regulated by the cell cycle (it initiates during late G1/S phase, peaks in

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G2/early mitosis and declines in late mitosis/early G1; reviewed in Laoukili *et al.* (2007) and Park *et al.* (2008)), FoxM1 also drives cell cycle progression by transactivating key G2/M regulatory genes, including polo-like kinase-1, survivin, centromere proteins A/B/F, aurora kinase B, S-phase kinase-associated protein-2, Csk1, cell division cycle 25 homolog B (*S. pombe*), cyclin B and NIMA (never in mitosis gene a)-related kinase 2 (Laoukili *et al.*, 2005; Wang *et al.*, 2005; Fu *et al.*, 2008). FoxM1 null mouse embryonic fibroblasts or human cells treated with small interfering RNA (siRNA) show reduced expression of such genes and increased levels of the cell cycle inhibitors p27 and p21 (Laoukili *et al.*, 2005; Wang *et al.*, 2005). Consequently, FoxM1-ablated cells experience a prolonged G2 phase and delays in mitotic entry (Laoukili *et al.*, 2005; Wonsey and Follettie, 2005).

FoxM1 is commonly upregulated in human carcinomas that originate from different tissues (Pilarsky *et al.*, 2004) and drives tumor development by stimulating proliferation (as reviewed in Laoukili *et al.*, 2007). FoxM1 expression is upregulated, and in many cases required, in several cancer types: basal cell carcinoma (Teh *et al.*, 2002), hepatocellular carcinoma (Kalinichenko *et al.*, 2004), glioblastoma (Liu *et al.*, 2006), primary breast cancer (Wonsey and Follettie, 2005), lung cancer (Kim *et al.*, 2006), prostate cancer (Kalin *et al.*, 2006) and gastric cancer (Zeng *et al.*, 2009). Furthermore, FoxM1 has been implicated in tumor formation (Gusarova *et al.*, 2007), angiogenesis (Wang *et al.*,

2007), metastasis (Chandran *et al.*, 2007) and inhibition of oxidative-stress-induced senescence through downregulation of the p19(Arf)-p53 pathway (Li *et al.*, 2008).

On the basis of the gene expression array data that FoxM1 levels inversely correlated with p53, our goal was to confirm that FoxM1 is a *bona fide* p53 repression target and, if so, to elucidate the mechanism and implications of such repression. In this study we show that pro-proliferative FoxM1 is indeed downregulated by the tumor suppressor p53 in multiple cell types after p53 stabilization or activation in a manner that involves the p21/Rb/E2F axis. The data provided in this paper also suggest that FoxM1 repression by p53 may contribute to and be critical for the long-term maintenance of the G2 arrest.

Results

FoxM1 mRNA is downregulated in a p53-dependent manner

To analyse the role of p53 in the regulation of FoxM1 mRNA levels, two cell lines that express tetracycline-regulated wild-type p53 were used. The H24 (Chen *et al.*, 1996; Baptiste *et al.*, 2002) and MCF7-24 cells (Zhu *et al.*, 2000), which are derivatives of H1299 and MCF7 cells, respectively, were grown in the presence or absence of tetracycline. The p53 induction after tetracycline withdrawal correlated with the downregulation of FoxM1 mRNA levels (Figure 1a). This suggests that

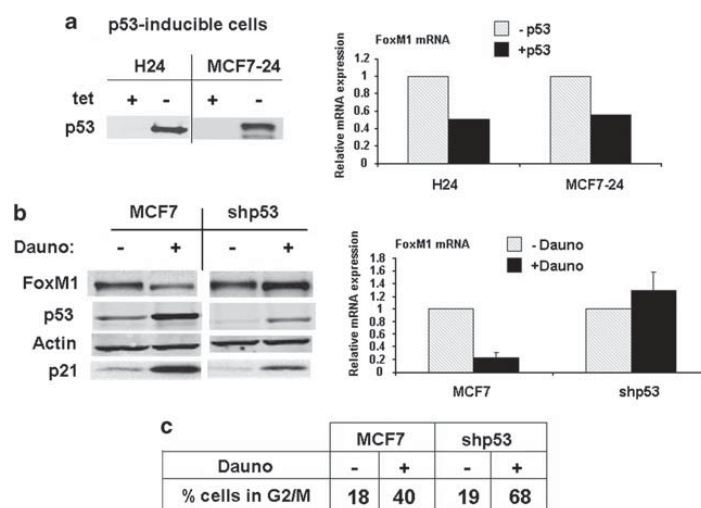


Figure 1 FoxM1 mRNA is downregulated in a p53-dependent manner. (a) Left panel: p53 was induced in H24 and in MCF7-24 cells for 48 h by withdrawal of tetracycline (tet) from the growth medium, and p53 in cell extracts was detected using immunoblotting. Right panel: relative FoxM1 mRNA levels were determined using quantitative reverse transcription-polymerase chain reaction (QRT-PCR) and were normalized to 1 for each + tet (-p53) condition (light gray, -p53; black, +p53). (b) MCF7 wild-type cells or MCF7 cells that express a short hairpin RNA (shRNA) to p53 (shp53) were either untreated or treated with daunorubicin (0.22 μ M) for 24 h. Left panel: immunoblot shows FoxM1, p53 and p21 levels with and without daunorubicin (dauno) treatment. As a loading control, extracts were probed for actin. Right panel: changes in FoxM1 mRNA levels were assessed using QRT-PCR in three independent experiments (light gray, -dauno; black, +dauno). Relative mRNA expression was normalized to 1 for each untreated (-dauno) condition. (c) The average percentage of cells in G2/M phases of the cell was obtained using fluorescence-activated cell sorting (FACS) analysis (also graphically represented in Figure 5a).

p53 expression, in the absence of any stimuli, facilitates FoxM1 transcriptional repression. p53 levels were also stabilized in MCF7 cells treated with daunorubicin, a DNA-damaging agent (Figure 1b), and in this study FoxM1 mRNA levels were repressed to a much greater extent (that is, by a factor of 5) after daunorubicin treatment, with an accompanying decrease in FoxM1 protein levels. MCF7 cells that constitutively express short hairpin RNA to p53 did not repress FoxM1 at either the mRNA or the protein levels, and in fact showed upregulation of FoxM1, confirming a p53-dependent mechanism. Furthermore, it is unlikely that the observed downregulation of FoxM1 is a secondary consequence of cell cycle arrest. Daunorubicin treatment caused arrest of both MCF7 wild-type and MCF7 shp53 cells in the G2/M phase of the cell cycle (Figure 1c). As FoxM1 levels are known to peak in these cell cycle phases, the downregulation of FoxM1 levels, specifically observed in the wild-type p53-expressing cells, is not due to p53-mediated cell cycle arrest at a point in the cell cycle when FoxM1 levels are inherently low.

Repression of FoxM1 is p21 dependent

As p21 is an important mediator of p53-dependent repression, we compared FoxM1 protein and mRNA levels in p53-null H1299 derivative cell lines engineered to express inducible ('tet-off') wild-type p53, p53^{Q22/S23} and p21 (Figure 2a). As before, FoxM1 was significantly repressed upon induction of wild-type p53. The two substituted amino acids in p53^{Q22/S23} are located within the first transactivation domain of p53 and render it largely impaired in its ability to induce p21 as well as many other p53 target genes (Baptiste *et al.*, 2002). The representative immunoblot shows that p53^{Q22/S23} accumulated to higher levels than wild-type p53, most likely because of its inability to transactivate murine double minute 2 (MDM2), the major negative regulator of p53 (Iwakuma and Lozano, 2003), and, as expected, only very weakly induced p21. Furthermore, p53^{Q22/S23} was unable to facilitate FoxM1 mRNA downregulation. Conversely, induction of p21 in the absence of p53 was sufficient for repression of FoxM1 mRNA. The magnitude of FoxM1 repression after p21 expression

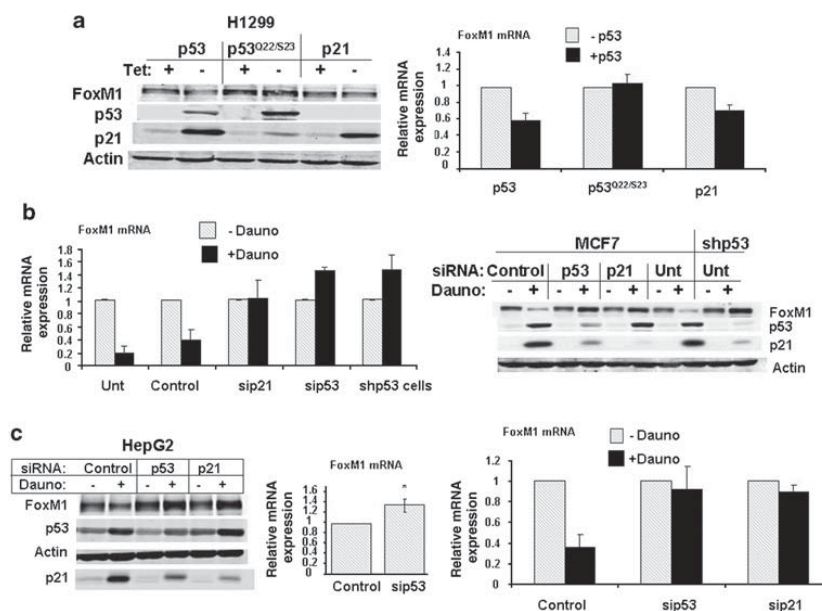


Figure 2 The repression of FoxM1 is p21 dependent. (a) H1299-derivative cell lines containing tetracycline (tet)-regulated wild-type p53 (H24 cell line), transcriptionally impaired p53^{Q22/S23} or p21 were grown in the presence or absence of tetracycline for 48 h before extraction for protein and RNA analysis. Left panel: immunoblot shows levels of FoxM1, p53, p21 proteins with actin as loading control. Right panel: relative FoxM1 mRNA expression was assessed using quantitative reverse transcription-polymerase chain reaction (QRT-PCR) and was normalized to 1 for each + tet condition (light gray, + tet (-p53 or p21); black, -tet, (+p53 or p21)). (b) MCF7 cells were treated with control, p53 or p21 small interfering RNA (siRNA) for 72 h. Daunorubicin (dauno, 0.22 μ M) was added for the final 24 h before extracting cells for protein and RNA analysis. MCF7 shp53-expressing cells were also treated with daunorubicin for 24 h, but were not transfected with siRNA (Unt, untransfected). Right panel: immunoblot shows FoxM1, p53 and p21 protein levels with actin shown as a loading control. Left panel: relative FoxM1 mRNA expression was assessed using QRT-PCR and was normalized to 1 for each untreated condition (-dauno), (light gray, -dauno; black, + dauno). (c) HepG2 cells were treated with control, p53, or p21 siRNA for 72 h and 0.22 μ M daunorubicin was added for the final 24 h before extraction of cells for protein and RNA analysis. Left panel: immunoblot shows levels of FoxM1, p53 and p21 with actin as a loading control. Middle panel: relative FoxM1 mRNA expression, assessed using QRT-PCR, was normalized to 1 for the untreated control siRNA condition and compared with FoxM1 mRNA levels in the untreated sip53 condition. Right panel: FoxM1 relative mRNA expression was assessed using QRT-PCR and was normalized to 1 for each untreated condition (-dauno) (light gray, -dauno; black, + dauno).

was comparable to, though slightly less than, that observed after expression of wild-type p53. Thus, induction of p21 is both necessary and sufficient for FoxM1 mRNA repression in H1299 cells.

To assess the importance of p21 levels induced in cells with endogenously expressed p53, MCF7 cells were treated with siRNA to p21 in combination with daunorubicin. As previously shown, MCF7 cells repressed FoxM1 mRNA and protein, whereas p53-knockdown cells induced FoxM1 levels in response to daunorubicin treatment (Figure 2b). Furthermore, MCF7 cells treated with siRNA versus p53 behaved identically to their shp53-expressing counterparts, ruling out clonal variation as an explanation for the lack of repression observed in cells with constitutively low p53 levels. Markedly, cells treated with siRNA versus p21 were unable to repress FoxM1 mRNA. In fact, under this condition, an increase in FoxM1 protein levels was observed, similar to what occurred in cells with reduced p53 expression. We conclude that p21 expression is necessary for FoxM1 repression in daunorubicin-treated MCF7 cells.

FoxM1 activity has been shown to be crucial to hepatocyte DNA replication and mitosis (Wang *et al.*, 2002). We therefore extended our studies to examine FoxM1 regulation in HepG2 cells (Figure 2c), a hepatocellular cancer cell line that harbors endogenous wild-type p53. Furthermore, HepG2 cells in which FoxM1 activity has been partially inhibited undergo significant apoptosis (Gusarova *et al.*, 2007). As shown

in Figure 2c, accumulation of p53 protein after daunorubicin treatment led to downregulation of FoxM1 mRNA levels in HepG2 cells (right panel). Cells treated with siRNA versus p53 or siRNA versus p21 were unable to repress FoxM1 message and stabilize FoxM1 protein levels as observed in MCF7 cells, confirming both p53 and p21-dependent repression of FoxM1 (right and left panels). Intriguingly, FoxM1 protein levels and mRNA levels were also somewhat increased after knockdown of basal p53 levels (left and middle panels, compare –dauno control siRNA with –dauno sip53), suggesting a role for basal levels of p53 in the maintenance of FoxM1 levels.

Retinoblastoma (Rb) family members have a role in FoxM1 mRNA repression

p21 exerts regulation of the cell cycle largely through inhibition of cyclin-dependent kinases and maintenance of Rb in a hypophosphorylated state, allowing Rb to complex with and inhibit E2F family members. The FoxM1 promoter contains two putative E2F-binding sites that are in close proximity to its transcription start site (Laoukili *et al.*, 2007). To determine whether the p21-dependent repression of FoxM1 observed in daunorubicin-treated MCF7 cells works through Rb family activation, MCF7 cells were treated with a combination of siRNA to three Rb family members (Rb, p130 and p107) (Figure 3a). As before, MCF7 cells treated with control siRNA repressed FoxM1 mRNA. MCF7 cells treated with siRNA to the Rb family members showed

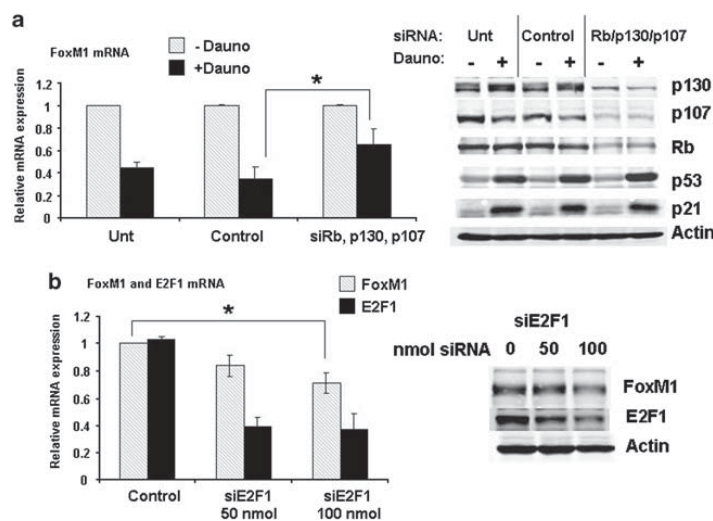


Figure 3 Retinoblastoma (Rb) family members have a role in FoxM1 mRNA repression (a) MCF7 cells were treated with a combination of small interfering RNA (siRNA) to Rb, p130 and p107 or control siRNA for 72 h and 0.22 μ M daunorubicin (dauno) was added for the final 24 h before extraction for protein and mRNA analysis. Left panel: relative FoxM1 mRNA expression was assessed using quantitative reverse transcription–polymerase chain reaction (QRT–PCR) and was normalized to 1 for each untreated condition (–dauno), (light gray, –dauno; black, +dauno). Right panel: immunoblot shows levels of p130, p107, Rb, p53 and p21 with actin as a loading control in untreated and daunorubicin-treated cells. (b) MCF7 cells were treated with either control or E2F1 siRNA at a final siRNA concentration of 100 nM for 48 h before extraction for RNA and protein analysis. Right panel: immunoblot shows FoxM1 and E2F1 proteins with actin as a loading control. Left panel: QRT–PCR was used to determine both FoxM1 and E2F1 relative mRNA expression (light gray, FoxM1 mRNA levels; black, E2F1 mRNA levels). Relative mRNA expression for each primer set was normalized to 1 for the control siRNA condition. Statistical significance ($P < 0.05$) is denoted with an asterisk.

partial abrogation of FoxM1 downregulation at the mRNA level (approximately twofold less repression was observed; Figure 3a). The complete rescue of repression may be unachievable because of incomplete knockdown of the three Rb family members, although it is possible that p21 acts through distinct pathways to inhibit activation of FoxM1.

To further analyse the involvement of the Rb family members in FoxM1 repression, we asked whether E2F1 can activate FoxM1. Depletion of E2F1 levels using two concentrations of siRNA caused decreasing FoxM1 mRNA and protein levels that were especially apparent at the highest amount of siRNA (Figure 3b). This suggests that E2F1 can exert control over FoxM1 expression, and that the canonical p53/p21/Rb/E2F1 pathway is likely to contribute to FoxM1 repression in daunorubicin-treated MCF7 cells.

Nutlin treatment extends the range of cell types showing p53-dependent repression of FoxM1

We extended our studies of FoxM1 to two other cancer cell lines, U2OS (osteosarcoma) and HCT116 (colon carcinoma) cells, and found that, in contrast to HepG2 and MCF7 cells, FoxM1 mRNA repression after daunorubicin treatment was very mild (Figures 4a and b). Such repression, although modest, was completely

reversed in HCT116 cells lacking either p53 or p21 (Figure 4b). In fact, a marked increase in FoxM1 protein levels was observed after daunorubicin in both HCT116 and U2OS cells, suggesting that DNA damage acts as a positive regulator of FoxM1 protein levels in the absence of substantial FoxM1 mRNA repression.

To determine whether these two cell lines were unable to effectively repress FoxM1 in any context, we chose to activate p53 by treating cells with nutlin-3, a small molecule that binds to and disrupts the interaction between MDM2 and p53 (Vassilev *et al.*, 2004). Nutlin inhibits both MDM2-dependent p53 ubiquitination and MDM2-dependent inhibition of p53-dependent transcription.

Stabilization of p53 by nutlin-3 led to a dramatic repression of FoxM1 at both the mRNA and protein levels in U2OS and HCT116 cells (Figures 4a and b), whereas HCT116 p53^{-/-} cells were unable to repress FoxM1 at the mRNA or protein level. On the other hand, nutlin-treated HCT116 p21^{-/-} cells showed mild FoxM1 mRNA repression. Yet, considering the strong repression observed in wild-type cells, nutlin-mediated repression can be considered to be largely p21 dependent.

As FoxM1 mRNA levels were indeed reduced in HCT116 p21^{-/-} cells after nutlin treatment, this suggests that p53 must be able to work in a p21-

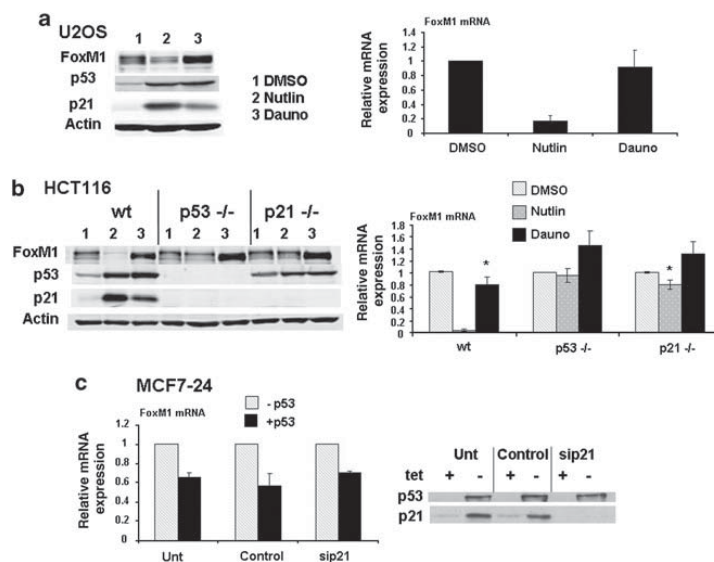


Figure 4 Nutlin is a potent FoxM1 repressor in two cancer cell lines that do not effectively repress FoxM1 in response to daunorubicin (dauno). (a and b) U2OS (osteosarcoma) or HCT116 (colon carcinoma) cells (wild-type, p53^{-/-}, p21^{-/-}) cells were treated with either dimethylsulphoxide (DMSO), 10 μ M nutlin-3 or 0.22 μ M daunorubicin for 24 h before extraction for RNA and protein analysis. Left panels: immunoblots show levels of FoxM1, p53 and p21 with actin as loading controls. Right panels (top, U2OS; bottom, HCT116) show relative FoxM1 mRNA levels determined using quantitative reverse transcription-polymerase chain reaction (QRT-PCR). The mRNA levels were normalized to 1 for the DMSO-treated condition (for HCT116, light gray, DMSO; dark gray, nutlin; Black, dauno) Statistical significance ($P < 0.05$) is denoted with an asterisk. (c) MCF7-24 cells were treated with control or p21 small interfering RNA (siRNA) for 72 h. At 48 h after siRNA transfection, tetracycline (tet) was withdrawn from the cell culture medium to allow for 24 h of p53 induction. Left panel: relative FoxM1 mRNA levels were assessed using QRT-PCR and were normalized to 1 for each + tet (-p53) condition, (light gray, -p53; black, +p53). Right panel: immunoblot shows p53 levels and p21 levels.

independent manner to repress FoxM1. Intriguingly, p21-independent repression of FoxM1 was also observed in the MCF7-24 tetracycline-regulated p53 cells (Figure 4c). These cells were pretreated with p21 siRNA with subsequent induction of p53. Although FoxM1 mRNA repression was not particularly strong after ectopic p53 expression in this cell line, the repression was largely, if not completely, p21-independent, a result in sharp contrast with the p21-dependent repression observed in MCF7 cells after DNA damage.

FoxM1 is required for proliferation of MCF7 cells, and stable G2 arrest of MCF7 cells may be facilitated by p53-dependent FoxM1 repression

To deduce the biological consequence of p53-dependent repression of FoxM1, we determined the outcome of FoxM1 ablation in MCF7 cells. The cells were treated with two different siRNAs that reduce FoxM1 levels to different extents, with FoxM1 siRNA no. 1 giving the most efficient knockdown (especially notable at the mRNA levels, data not shown). After treatment with

either of the FoxM1 siRNAs, the MCF7 cells arrested with 4N content, assessed using fluorescence-activated cell sorting analysis, indicative of cells arrested at G2/M phase of the cell cycle (Figure 5a). Furthermore, MCF7 cells expressing shp53 also arrested in G2/M phase of the cell cycle, ruling out the possibility that the arrest observed after siFoxM1 was due to p53 activation. This suggests that FoxM1 is necessary for proper passage of MCF7 cells through G2.

The extent of G2/M arrest observed after FoxM1 siRNA no. 1 was similar to that observed after daunorubicin treatment of untransfected MCF7 cells. As such, the contribution of p53-mediated repression of FoxM1 to G2/M arrest was assessed. However, even cells expressing shp53 arrested in G2/M in response to daunorubicin (Figure 5a). In fact, these cells arrested to a greater extent in G2 than did p53-expressing cells because of an inability to retain cells in G1, a function ascribed primarily to the action of p53. The observation that shp53-expressing cells arrest in G2 is not surprising as p53 is not thought to be necessary for initial G2/M arrest (Kastan *et al.*, 1991; Bunz *et al.*, 1998; Taylor and Stark, 2001).

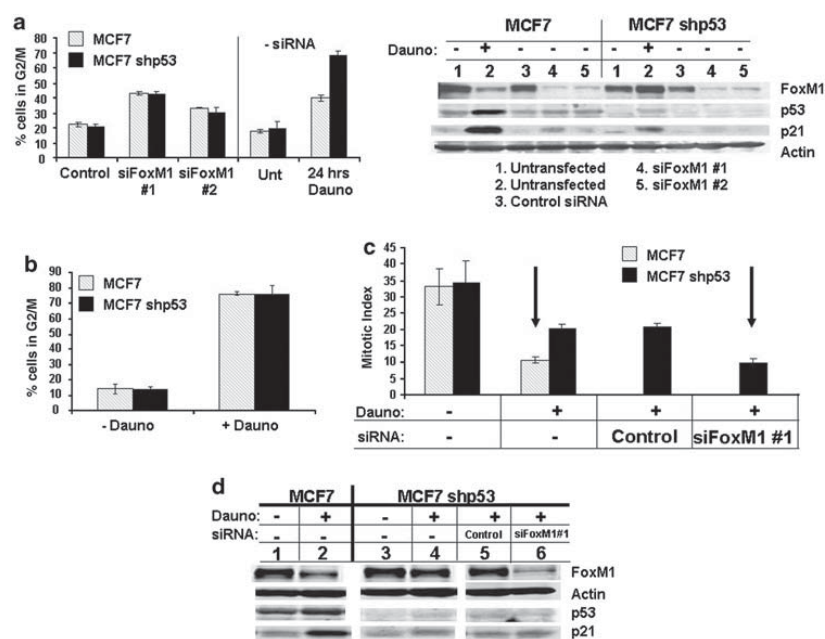


Figure 5 The p53-mediated repression of FoxM1 has a role in stable G2 arrest. (a) MCF7 wild-type and shp53-expressing cells were treated with control, FoxM1 no. 1, or FoxM1 no. 2 small interfering RNA (siRNA) for 72 h. Alternatively, cells were untransfected (Unt) but treated with daunorubicin (dauno, 0.22 μ M) for 24 h. Left panel: cells were subjected to fluorescence-activated cell sorting (FACS) analysis and the percentage of cells with a 4N content in each of the above-mentioned conditions was determined and plotted on the y axis of the graph as percent G2/M. Right panel: representative immunoblot shows FoxM1, p53 and p21 protein levels after siRNA or daunorubicin treatment. Cells were untransfected (lane 1), untransfected but treated with daunorubicin (lane 2), treated with control siRNA (lane 3) or treated with siRNA no. 1 (lane 4) or siRNA no. 2 (lane 5) versus FoxM1. (b) MCF7 wild-type and shp53-expressing cells were treated for 2 h with 0.22 μ M daunorubicin, which was washed out, and cells were allowed to recover for 72 h. FACS analysis was performed and the percentage of cells with a 4N content is plotted on the y axis of the graph as percent G2/M. (c) The same time course/treatments as in (b) were applied with the addition of taxol for the final 20 h of the time course to trap cells in mitosis. The mitotic index was determined by the percentage of cells that were positive for phosphorylated ser10H3 as assessed using FACS analysis. (d) Immunoblot analysis of cell extracts of replicate cultures corresponding to the conditions in (c). -/+ acute daunorubicin treatment + taxol. In all graphs in this figure, light gray bars represent wild-type MCF7 cells and black bars represent MCF7 shp53 cells.

To evaluate the contribution of FoxM1 repression to arrest, we assayed for the ability of MCF7 cells and MCF7 shp53-expressing cells to maintain a stable G2 arrest after acute daunorubicin treatment by modifying a previously described approach (Bunz *et al.*, 1998). Both cell lines were treated with daunorubicin for 2 h and allowed to recover for 72 h after removal of the drug (Figure 5b). In a subsequent experiment, cells were treated with daunorubicin as above and also trapped in mitosis by the addition of taxol (Figure 5c), a mitotic poison that prevents the depolymerization of microtubules. In the latter case, mitotic indexes were calculated based on the percentage of cells expressing histone H3 that has been phosphorylated at Serine 10, a mark closely associated with the condensation of chromatin observed during mitosis (Hendzel *et al.*, 1997).

Both MCF7 wild-type and shp53-expressing cells were strongly arrested to similar levels in G2/M, 72 h after acute daunorubicin treatment (in the absence of taxol, Figure 5b). Despite similar extents of G2 arrest, shp53-expressing cells entered mitosis at a significantly higher rate than cells that retained wild-type p53 (a twofold greater mitotic index, Figure 5c). These data show that p53 has a role in the long-term maintenance of G2 arrest after DNA damage.

To determine the possible contribution of p53-mediated repression of FoxM1 in the stability of G2 arrest, shp53-expressing cells (which fail to repress FoxM1 after daunorubicin treatment) were treated with FoxM1 siRNA no. 1 before daunorubicin treatment. Strikingly, ablation of FoxM1 completely restored the mitotic index of shp53-expressing cells to the same level as that observed for daunorubicin-treated wild-type p53 cells (Figure 5c, compare bars indicated by arrows). Daunorubicin caused FoxM1 repression in MCF7 cells but not in shp53 cells (Figures 1b and 2b), suggesting that p53-mediated repression of FoxM1 can contribute to and is likely to be important for maintenance of a stable G2 arrest.

In this particular assay, the acute daunorubicin treatment yielded greater than twofold FoxM1 mRNA repression (data not shown), but did not give rise to the strong downregulation of FoxM1 protein observed in Figures 1b and 2b, most likely because of the stabilizing effect of DNA damage on FoxM1 protein. Nonetheless, the negative regulation of FoxM1 protein levels can be clearly shown after taxol treatment (Figure 5d). In general, FoxM1 levels increased after taxol treatment (data not shown). Despite the high levels of FoxM1, the activation of p53/p21 after acute daunorubicin treatment resulted in significantly lower levels of FoxM1 (lane 2) compared with both untreated wild-type cells (lane 1) and shp53-expressing cells with and without daunorubicin treatment (lanes 3 and 4).

Discussion

This study demonstrates that FoxM1, an essential transcription factor that controls the expression of

many G2/M target genes, is downregulated by p53. Although ectopic p53 expression results in a reduction of FoxM1 mRNA levels, DNA damage cooperates with p53 (perhaps through modification of p53) to more potently repress FoxM1 mRNA. Nonetheless, DNA damage in the relative absence of p53 (shp53) gives rise to an increase in both FoxM1 mRNA and protein over basal levels, supporting previous work that identifies DNA damage as a positive regulator of FoxM1 protein stability (Tan *et al.*, 2007). Thus, DNA damage seems to cause the activation of multiple signaling pathways that converge to fine-tune FoxM1 levels.

As has been found for multiple targets of p53-mediated repression (Kannan *et al.*, 2001; Lohr *et al.*, 2003; Shats *et al.*, 2004), our data in MCF7, HepG2 and H1299 cells establish FoxM1 as an indirect repression target in which downregulation depends on p21. The Rb family (Rb, p130, p107) operates downstream of p21 and has been implicated in p53-mediated repression (Gottifredi *et al.*, 2001; Taylor *et al.*, 2001; Shats *et al.*, 2004; Jackson *et al.*, 2005). Similar to some FoxM1 target genes including Plk1 (Jackson *et al.*, 2005), our results indicate that the Rb family has a role in FoxM1 repression. Although it is unknown whether E2F1 directly activates FoxM1, two putative E2F1 sites have previously been identified in the FoxM1 promoter (Laoukili *et al.*, 2007). The finding that E2F1 contributes to FoxM1 expression further implicates Rb's involvement in FoxM1 repression.

An intriguing possibility is that p21 may also function to inhibit other transcription factors that are responsible for FoxM1 activation. The FoxM1 promoter contains multiple putative transcription factor binding sites, and has been shown to be downstream of both Gli1 (Teh *et al.*, 2002) and c-Myc (Fernandez *et al.*, 2003; Blanco-Bose *et al.*, 2008) transcription factors. Interestingly, p21 is known to inhibit c-Myc-dependent transcription through direct interaction that disrupts the c-Myc-Max complex (Kitauro *et al.*, 2000). In addition to putative E2F1-binding sites, the FoxM1 promoter contains a B-Myb binding site and the *cis*-regulatory module CHR-NF-Y (Linhart *et al.*, 2005). p21 could again participate here by impairing NF-Y function through inhibition of cyclin-dependent kinase 2 (Yun *et al.*, 1999, 2003). Furthermore, the presence of CDE/CHR elements in a gene's promoter often correlates with an indirect repression by p53 (Badie *et al.*, 2000; St Clair *et al.*, 2004). In addition, our data reveal that a portion of FoxM1 repression can be considered p21 independent in both nutlin-treated HCT116 cells and after ectopic p53 expression in MCF7-24 cells. Although the mechanism is unexplored, p53-dependent inhibition of the factors listed above could effectuate this repression. In fact, a well-characterized interaction between p53 and NF-Y is known to directly inhibit NF-Y-dependent transcription (Imbriano *et al.*, 2005). Alternatively, the induction of microRNAs by p53 (reviewed in He *et al.* (2007) and Vousden and Prives (2009)) could lead to the observed p21-independent FoxM1 repression.

We observe that the basal levels of p53 contribute to FoxM1 regulation in HepG2 cells. This reflects the

findings of a study in which p53 represses expression of the cell-surface molecule CD44 under basal conditions (Godar *et al.*, 2008), allowing cells to respond to apoptotic signals that would otherwise be blocked by CD44. Similarly, survivin (a FoxM1 target gene), has been shown to be regulated by the basal levels of p53 and Rb (Raj *et al.*, 2008). As FoxM1 is implicated in negative regulation of the cell cycle inhibitors, p21 and p27 (Wang *et al.*, 2005, 2007; Chan *et al.*, 2008; Xia *et al.*, 2008; Penzo *et al.*, 2009), repression of FoxM1 might be necessary for full p53-dependent responses to stress/treatments.

Furthermore, as loss of p53 and Rb are frequent occurrences in tumors, their absence may contribute to FoxM1 misregulation and thereby adversely affect the ability to inhibit cellular proliferation. In fact, while this manuscript was being prepared, one report showed that p21 (which we have shown to be an important mediator of p53-dependent FoxM1 repression) is required for proper FoxM1 suppression during dextrose-mediated inhibition of liver regeneration after partial hepatectomy (Weymann *et al.*, 2009). This finding confirms the importance of p21 in FoxM1 regulation and also highlights a biological context in which precise regulation of FoxM1 levels is crucial to inhibition of proliferation. As such, it is likely that the inability to downregulate FoxM1 after loss of the tumor suppressor p53 will contribute greatly to biological processes such as carcinogenesis.

Although DNA damage facilitated FoxM1 repression in MCF7 and HepG2 cells, two other cell lines (U2OS and HCT116) were surprisingly unable to effectively repress FoxM1 mRNA and stabilize FoxM1 protein levels after daunorubicin treatment. This finding is reminiscent of a study in which doxorubicin caused p53/Rb-dependent downregulation of human telomerase reverse transcriptase in MCF7 but not in HCT116 cells (Shats *et al.*, 2004). However, we show that nutlin-3 does cause notable downregulation of FoxM1 mRNA and protein in both HCT116 and U2OS cells. Nutlin-3 activates p53 in the absence of DNA-damage signaling by disrupting the interaction between p53 and MDM2 (its major negative regulator). As disruption of the p53-MDM2 complex is thought to be complete after nutlin treatment, it is possible that residual MDM2-p53 complexes remaining after ectopic p53 expression or after DNA damage curb the ability of p53 to downregulate FoxM1. Alternatively, these particular cell lines may require high p21 levels observed only after nutlin treatment (Figures 4a and b) to repress FoxM1 (and possibly other indirect targets such as human telomerase reverse transcriptase).

As FoxM1 is often overexpressed in cancers, the biological outcome of p53-mediated repression of FoxM1 was an important goal of this study. In MCF7 cells, siRNA to FoxM1 causes a p53-independent G2 arrest. This underscores the importance of FoxM1 and supports a rich literature that depicts FoxM1 as a pro-proliferative transcription factor. As p53 is a major regulator of the cell cycle, it was of great interest to determine the contribution of FoxM1 repression to decisions about cell fate—namely, how does FoxM1 repression affect the cell cycle?

Although only cells that retain wild-type p53 repress FoxM1, this repression cannot be a requirement for initial G2 arrest, as both MCF7 wild-type and shp53-expressing cells arrest in G2 after DNA damage (Figure 5a). However, p53 is thought to be more important for maintenance of G2 arrest rather than its initiation (Taylor and Stark, 2001), as cells without p53 aberrantly enter mitosis with damaged DNA (an event that could lead to chromosomal aberrations, aneuploidy and potentially give rise to pro-tumorigenic cells). The Rb family members have also been shown to be important for a stable G2 arrest and cell cycle exit from G2 (Jackson *et al.*, 2005). On the contrary, FoxM1 has been shown to be important for entry into mitosis in both mouse cells and human osteosarcoma cells (U2OS cells) (Wang *et al.*, 2005).

The finding that FoxM1 siRNA rescues the aberrant mitotic entry of MCF7 shp53-expressing cells reveals the role of p53-mediated repression of FoxM1. That is, for conditions in which p53-mediated repression is robust, the downregulation of a single target, FoxM1, could facilitate stable G2 arrest. Although it is likely that p53 uses several pathways to maintain a stable G2 arrest, the repression of FoxM1 provides an elegant way for p53 to achieve global cellular changes. Notably, many reported targets of p53-mediated repression (Jackson *et al.*, 2005; Spurgers *et al.*, 2006) overlap with the set of genes induced by FoxM1 (Laoukili *et al.*, 2005, 2007; Wang *et al.*, 2005; Fu *et al.*, 2008). As FoxM1 is a master regulator of factors that facilitate the G2/M transition and regulate mitotic events, p53-mediated repression of FoxM1 may cause indirect negative regulation of such factors, ultimately leading to stable arrest and maintenance of genomic integrity.

Materials and methods

Cell culture and transfections

The HepG2 cells were maintained in RPMI/10% fetal bovine serum. All other cells were maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum. H24 (Baptiste *et al.*, 2002) and MCF724 cells (Zhu *et al.*, 2000) are derivatives of the H1299 and MCF7 cell lines, respectively, that are engineered to inducibly express either wild-type p53, p53^{Q22/S23} or p21 under control of tetracycline-regulated promoters ('tet-off'). MCF7 cells that stably express a short hairpin RNA to p53 (MCF7 shp53-expressing cells) were grown in Dulbecco's modified Eagle's medium/10% fetal bovine serum with 2.5 µg/ml puromycin. Both MCF7-derivative cell lines were kindly provided by Dr Xinbin Chen. HCT116 wild-type cells and their derivatives HCT116 p53^{-/-} and p21^{-/-} were generously provided by B Vogelstein. MCF7 (mammary carcinoma), HepG2 (hepatocellular carcinoma), U2OS (osteosarcoma) and HCT116 (colorectal carcinoma) cells were treated with daunorubicin (0.22 µM; Oncogene Research Products, La Jolla, CA, USA) or nutlin-3 (10 µM; Sigma-Aldrich, St Louis, MO, USA) for 24 h unless otherwise noted.

The siRNA duplexes from Qiagen (Valencia, CA, USA) were used individually at 50 nmol unless otherwise noted, and transfected into cells using Dharmafect 1 reagent (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. Control siRNA (either Qiagen All-Stars or siLuciferase)

represented a negative control siRNA. The RNA oligo sequences were as follows: siFoxM1 no. 1 (published as siFoxM1 no. 2 in Wang *et al.*, 2005) GGACCACUUUCCCUACUUUdTdT, siFoxM1 no. 2 (Wonsey and Follettie, 2005) CCUUUCCCU GCACGACAUGdTdT, sip21 CUUCGACUUUGUCACCGA GdTdT, sip53 CUACUUCUGAAAACAACGdTdT, sip130 (Jackson and Pereira-Smith, 2006) GAGCAGAGCUUAAUC GAAUUU, sip107 (Jackson and Pereira-Smith, 2006) CAAG AGAAGUUGUGGCAUAAU, siE2F1 GUCACGCUAUG AGACCUAdTdT, siRB AAGATACCAGATCATGTCAGA siLuciferaseGL3 CTTACGCTGAGTACTTCGAdTdT.

Western blot analysis

Cell extracts were analysed according to standard western blotting procedures using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) or fluorescence through use of the Odyssey system (LI-COR, Lincoln, NE, USA). The monoclonal antibodies DO1 or 1801 were used to detect p53. The monoclonal antibody XZ131 was used to detect Rb. The following polyclonal antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA): anti-FoxM1 (C-20) sc-502, anti-p21 (C-19) sc-397, anti-p130 (C-20) sc-317, anti-p107 (C-18) sc-318, and anti-E2F1 (C-20) sc-193. Anti-Actin (A2066) was purchased from Sigma (St Louis, MO, USA).

Quantitative reverse transcription-polymerase chain reaction to assess relative mRNA levels

Quantitative reverse transcription-polymerase chain reaction was used to quantify relative changes in mRNA expression. RNA was isolated from cell cultures using the Qiagen RNeasy Mini Kit. Complementary DNA was amplified using Qiagen Quantitect reverse transcription kit (Qiagen, Valencia, CA, USA). PCR was carried out on an Applied Biosystems Prism 7300 using the SYBR green dye (Applied Biosystems, Foster City, CA, USA). The mRNA expression was assayed in triplicate and normalized to RPL32 mRNA expression. The relative levels were calculated using the Comparative-Ct Method ($\Delta\Delta C_T$ method). Primers were designed with *Primer Express* (Applied Biosystems): RPL32-F 5'-TTCCTGGCTCA CAACGTCAAG-3', RPL32-R 5'-TGTGAGGGATCTCGG CAC-3', FoxM1-F 5'-TGCCCAGATGTGCGCTATTA-3', FoxM1-R 5'-TCAATGCCAGTCTCCCTGGTA-3', E2F1-F 5'-AGATGGTTATGGTGATCAAAGCC-3', E2F1-R 5'-AT CTGAAAGTTCTCCGAAGAGTCC-3'.

The error bars were derived from the s.d. of multiple experiments. Levels of significance were calculated using the Student's *t*-test. Statistical significance ($P < 0.05$) is denoted with an asterisk.

Flow cytometry analysis

Cells pellets were washed with phosphate buffered saline and fixed/permeabilized with 50% ice-cold ethanol. Pellets were washed and resuspended in 50 μ g/ml ribonuclease A and 62.5 μ g/ml propidium iodide. Samples were analysed on the Becton Dickinson FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). The percentages of cells in various phases of the cell cycle were quantified using the ModFit LT Version 3.0 program (Verity Software House, Topsham, ME, USA). The error bars were derived from the s.d. of multiple experiments.

Determination of mitotic index

To determine the mitotic index, the Phospho-Histone H3 (Ser10) Antibody (Alexa Fluor 488 Conjugate) (no. 9708) from Cell Signaling Technology (Danvers, MA, USA) was used. The manufacturer's recommended flow cytometry protocol for intracellular staining using conjugated primary antibodies (Cell Signaling) was followed with slight modification (cells were permeabilized in 50% ethanol). The samples were analysed for positive incorporation of the Alexa Fluor 488 conjugated phospho-histone H3 (Ser10) antibody on the FACSCalibur (Becton Dickinson). Mitotic index was calculated by dividing the number of Alexa Fluor 488 positive-staining cells with the total cells counted (as assessed by propidium iodide staining). Error bars were derived from the s.d. of multiple experiments.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 3

p53-dependent induction of *PVT1* and miR-1204

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Abstract

p53 is a tumor suppressor protein that acts as a transcription factor to regulate (either positively and negatively) a plethora of downstream target genes. While p53's ability to induce protein coding genes is well-documented, recent studies have implicated p53 in the regulation of non-coding RNAs, including both microRNAs (e.g. miR-34a), and long non-coding RNAs (e.g. lincRNA-p21). Like certain p53-inducible proteins (e.g. p21), non-coding RNAs often mediate downstream inhibition of gene expression. Thus, the functional connection between p53 and non-coding RNAs has brought the potential importance of indirect gene repression into sharp focus. We have identified the non-protein coding locus *PVT1* as a p53-inducible target gene. *PVT1* is a very large (>300 kb) locus located downstream of *c-Myc* on chromosome 8q24. *PVT1* produces a wide variety of spliced non-coding RNAs, as well as a cluster of 6 annotated microRNAs: miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p and miR-1208. Chromatin immunoprecipitation (ChIP), electrophoretic mobility shift assay (EMSA) and luciferase-assays reveal that p53 binds and activates a canonical response element within the vicinity of miR-1204. Consistently, we demonstrate the p53-dependent induction of endogenous *PVT1* transcripts (spliced non-coding RNA, primary microRNAs), leading to the up-regulation of mature miR-1204. Finally, we have shown that ectopic expression of miR-1204 reduces cell viability through either an induction of cell death or cell cycle arrest. As such, we believe that miR-1204 represents a functional target of p53 at the *PVT1* locus.

Introduction

p53 is a tumor suppressor protein whose function is inactivated (by mutations or by other means) with a high frequency in a wide spectrum of human cancers (Petitjean et al., 2007; Soussi, 2007). Several functions of p53 have been shown to contribute to its tumor suppressive function. For example, the ability of p53 to cause cell cycle arrest, senescence and cell death afford protection against carcinogenesis (Lozano, 2010; Vousden and Prives, 2009). p53 has also been shown to modulate diverse cellular processes, including (but not limited to) metabolic regulation, antioxidant defense, DNA repair, autophagy and differentiation (Levine and Oren, 2009; Vousden and Prives, 2009).

To achieve such cellular programs, p53 predominantly acts a sequence-specific transcription factor. In response to cellular stress, various signaling pathways converge to both stabilize p53 levels and direct its transcriptional activity toward specific target genes (Vousden and Prives, 2009). The protein products of many such target genes directly participate in processes that arrest cell cycle proliferation or result in apoptosis. For example, p21/*CDKN1A* (the first p53 target gene to be identified) directly inhibits cyclin-dependent kinases, thus halting progression through the cell cycle (Brugarolas et al., 1998; el-Deiry et al., 1994; el-Deiry et al., 1993). *PUMA* and *Noxa*, on the other hand, are p53-inducible targets that represent pro-apoptotic members of the Bcl-2 family that function by promoting Bax oligomerization and subsequent mitochondrial membrane permeabilization- an initiating event for the intrinsic apoptosis cascade (Yu and Zhang, 2005).

In addition to genes encoding protein products, p53 also induces transcription from non-protein coding genomic loci, including both microRNAs and long intergenic non-coding RNAs (lincRNAs). The biogenesis of microRNAs begins with RNA Polymerase-II-mediated transcription of a long primary RNA species (the primary microRNA) that is distinguished from other non-coding RNAs by the presence of characteristic stem-loop structures (Bartel, 2009). Consistent with the similarities between mRNA and primary microRNA transcription (Pol II-transcribed, 5'capping, 3'polyadenylation) (Cai et al., 2004), p53 has been shown to regulate transcription of primary microRNAs through binding of canonical p53 response elements (Shi et al., 2010). After several processing steps, the mature microRNA is finally incorporated into the RISC complex and silences gene expression through targeting mRNA for degradation or translational inhibition (Bartel, 2004; Lee et al., 2002; Zeng and Cullen, 2003). Long non-coding RNA represents a species of RNA greater than 200 nucleotides in length that is transcribed and processed similarly to mRNA, but lacks open reading frames that are translated into protein. Such RNA species may function in diverse ways, but are often involved in complexes with proteins that establish compacted chromatin states, and thus negatively affect transcriptional activity at select genomic regions (Khalil et al., 2009).

Just as is the case for protein-coding genes, the regulation of non-protein coding genes by p53 is also thought to contribute to p53's ability to modulate various cellular processes. For example, p53-inducible miR-34a participates in cell cycle arrest, senescence and apoptosis. (Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tazawa et al., 2007). To mediate such processes, miR-34a inhibits multiple mRNAs whose protein products inhibit apoptosis (e.g. Bcl-2), promote cell cycle

progression (e.g. b-Myb) or inhibit p53 activity (e.g. SIRT1) (Hermeking, 2007, 2010; Yamakuchi et al., 2008). p53 has also been shown to induce the transcription of various other microRNAs, including miR-145, miR-192 and miR-107. These microRNAs have been demonstrated to counteract cell cycle progression, pluripotency, and angiogenesis through the regulation of mRNAs such as c-Myc, Oct4/Sox2/Klf4, dihydrofolate reductase, and hypoxia-inducible factor 1-alpha (Georges et al., 2008; Sachdeva et al., 2009; Song et al., 2008; Xu et al., 2009; Yamakuchi et al., 2010). Recent work has revealed that p53 also induces the transcription of select long non-coding RNAs such as lincRNA-p21, which represents a key mediator of p53-dependent repression of pro-survival genes (Barsotti and Prives, 2010; Huarte et al., 2010).

The *PVT1* gene represents a non-protein coding locus that yields a wide variety of non-coding RNAs, including a cluster of six microRNAs. In addition, extensive alternative splicing produces a complex array of mature transcripts that may function independently of the *PVT1*-encoded microRNAs. Despite the complex regulation of *PVT1* RNAs, no protein product has yet been identified (Beck-Engeser et al., 2008; Huppi et al., 2008).

Importantly, many studies have implicated the *PVT1* genomic region in cancer biology. *PVT1* is a large (>300 kb) locus located adjacent to the *c-Myc* locus on human chromosome 8q24 (mouse chromosome 15). Similar to *c-Myc*, *PVT1* is frequently the target of retroviral integrations. Furthermore, translocation breakpoints within either the *c-Myc* or *PVT1* locus occur with a high frequency and are the characteristic lesions associated with Burkitt's lymphoma and mouse plasmacytomas (Beck-Engeser et al., 2008) (Huppi et al., 2008).

In the majority of Burkitt's lymphoma (~80%), translocations within the *c-Myc* locus juxtapose *c-Myc* to the immunoglobulin heavy chain. In contrast, *PVT1* reciprocal translocations (variant Burkitt's lymphoma, ~20%) fuse *PVT1* to the immunoglobulin light chain, either lambda (chromosome 22) or kappa (chromosome 2) (Beck-Engeser et al., 2008; Huppi et al., 2008; Zeidler et al., 1994). Despite this, these distinct translocation events produce indistinguishable disease phenotypes (Siwarski et al., 1997). The translocations observed in variant Burkitt's lymphoma give rise to abundant expression (due to proximity of the immunoglobulin enhancer) of fusion RNA transcripts containing either Exon 1A or alternative Exon 1B fused to the constant region of the immunoglobulin light chain (Huppi and Siwarski, 1994; Shtivelman and Bishop, 1990). Despite high expression levels, no protein product from these transcripts has been identified (Huppi et al., 2008). In addition, two unrelated *PVT1* fusion transcripts have also been described in lung cancer: *PVT1* exons 1-3 fused to exon 4-38 of *CHD7* and *PVT1* exon 1 fused to exons 14-38 of *CDH7* (Campbell et al., 2008; Pleasance et al., 2010).

PVT1 is amplified and/or overexpressed in multiple cancer types in which no *PVT1* translocations have been detected. In fact, numerous studies conducting global analysis of copy number alterations and gene expression changes in human tumors have identified *PVT1* as a candidate oncogene. *PVT1* copy number gain (e.g. double-minutes, amplifications) or overexpression of *PVT1* has been demonstrated in breast cancer, ovarian cancer, pediatric malignant astrocytomas, acute myeloid leukemia and hodgkins lymphoma (Enciso-Mora et al., 2010; Guan et al., 2007; Haverty et al., 2009; Kamath et al., 2008; Schiffman et al., 2010; Sircoulomb et al., 2010). In congruence with such

observations, *PVT1* expression has been reported to be low in normal tissue, but highly expressed in many transformed cell lines (Carramusa et al., 2007).

Further work has demonstrated that high levels of *PVT1* contribute to tumor biology. For example, multiple myeloma patients with rearrangement of the *PVT1* region are refractory to therapy (Palumbo et al., 1990). Coamplification of *c-Myc* and *PVT1* has been linked to poor clinical survival in certain breast cancer patients (e.g, postmenopausal or HER2-positive breast cancer patients) (Borg et al., 1992) and ovarian cancer patients (Guan et al., 2007). Furthermore, siRNA-mediated knockdown of *PVT1* selectively kills ovarian cancer cell lines with overexpressed *PVT1*. Inhibition of c-Myc in c-Myc overexpressing cancer cells, on the other hand, did not cause apoptosis (but did inhibit proliferation), suggesting a specific contribution of *PVT1* ncRNA to tumor survival (Guan et al., 2007).

Notably, overexpression of *PVT1* has also been linked to chemoresistance. A genome-wide screen identified *PVT1* as a cellular determinant that regulates sensitivity to the chemotherapeutic gemcitabine in pancreatic cancer (You et al., 2011). That is, the expression of full-length antisense *PVT1* RNA causes cell death of pancreatic cancer cells (ASPC-1) in response to gemcitabine-treatment, whereas overexpression of the full length sense-*PVT1* cDNA provided these cells with further protection. However, the precise *PVT1*-encoded RNA that provides gemcitabine- resistance remains unknown, as the authors attempts to isolate full length *PVT1* cDNA by RT-PCR resulted only in short isoforms of *PVT1* cDNA (less than 1 kb in length), but no full-length cDNA (~1.9 kb, NR_003367.1) (You et al., 2011).

Without a protein product or consensus functional long non-coding RNA, the results of the experiments described above have been difficult to interpret. A study in 2008 identified seven microRNAs encoded by the *PVT1* locus, six of which are annotated in miRBase (Huppi et al., 2008). The characterization of these microRNAs may provide otherwise elusive mechanistic insights into biological functions of *PVT1*.

The six annotated microRNAs of the *PVT1* locus are as follows: miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p and miR-1208 (Beck-Engeser et al., 2008; Huppi et al., 2008). All such microRNAs are located in intronic regions, with the exception of miR-1204 which overlaps *PVT1* alternative exon 1B. The mature form of these microRNAs are expressed to varying extents in several cancer cell lines, including Burkitt's lymphoma, breast and colon cancer cell lines (Huppi et al., 2008). miR-1207-5p was also reported to be among the top 30 overexpressed microRNAs in a collection of colon cancer specimens (Yamakuchi et al., 2010).

The function of the *PVT1*-encoded microRNAs has been largely unexplored. Suggesting a potential role for these microRNAs in B-cell development or even lymphoma, the precursor transcripts of all *PVT1*-encoded microRNAs increase with the stage of B-cell development (higher expression in late-stage B cells including variant Burkitt lymphoma cell lines and plasmacytoma cell lines) (Huppi et al., 2008). In addition, a recent study identified miR-1204 as a microRNA highly induced during the process of replicative senescence of normal human fibroblasts (Marasa et al., 2010). Aside from these findings, the function and regulation of *PVT1*-encoded microRNAs are largely unknown.

Given the frequency of alterations and contribution of *PVT1* to human cancer and the ability of p53 to transcriptionally regulate non-coding RNAs, we have explored the potential relationship between the tumor suppressor p53 and the *PVT1* locus. While *PVT1* pro-tumorigenic roles have been described, surprisingly we have found that p53 acts to positively regulate transcription of this locus through a canonical p53-binding site. Consistent with a potential role for miR-1204 in senescence (Marasa et al., 2010) (a p53-mediated cellular program (Qian and Chen, 2010)), this study also reveals that *PVT1*-encoded miR-1204 is a target of p53 that may contribute to cell death and cell cycle arrest.

Results

p53 induces transcription from the PVT1 locus

To investigate the potential link between the tumor suppressor p53 and the *PVT1* locus, we employed the isogenic colon carcinoma HCT116 and HCT116 p53^{-/-} cell lines. HCT116 cells harbor wild-type p53 whose levels can be stabilized by a variety of mechanisms, including cellular stress (e.g. DNA-damage) or molecules that specifically inhibit MDM2, the major negative regulator of p53 stability (Figure 1A). In HCT116 cells, activation of p53 by one such compound, Nutlin, led to increased RNA levels of the *PVT1* non-coding RNA (*PVT1* ncRNA) (Figure 1B). The DNA-damaging agent Daunorubicin also caused an upregulation of *PVT1* ncRNA, to an even greater extent than observed after Nutlin. Importantly, both Nutlin and Daunorubicin caused p53-dependent induction of *PVT1* ncRNA, as HCT116 p53^{-/-} cells either failed to induce, or

showed only partial induction, of *PVT1* ncRNA. Notably, the basal steady-state levels of *PVT1* ncRNA are also reduced in HCT116 p53^{-/-} cells.

As previously mentioned, *PVT1* ncRNA undergoes extensive alternative splicing to produce a wide variety of mature transcripts. Figure 1B represents the induction of *PVT1* ncRNA as measured by the RNA levels of exon 4, an internal exon of *PVT1*. The fold change of *PVT1* ncRNA induction as measured by other internal *PVT1* exons was consistent with those changes observed for exon 4-containing RNA molecules (data not shown). However, a difference in exon usage after p53-dependent induction of *PVT1* ncRNA can be delineated through the use of PCR primers that differentiate between the alternative exons 1A and 1B. Daunorubicin treatment led to a greater fold induction of Exon 1B than Exon 1A (Figure 1C). Nonetheless, the increase in both Exon 1A and Exon 1B-containing transcripts was abrogated in the absence of p53 (Figure 1C).

p53 binds a canonical response element at the PVT1 locus

Given our observation that *PVT1* ncRNA is induced in a p53-dependent manner, we investigated the mechanism by which p53 upregulates *PVT1* ncRNA. To this end, we identified a putative p53-binding site using the p53 MH algorithm (Hoh et al., 2002) which closely adheres to the p53 canonical consensus binding sequence. Depicted in Figure 2A, this binding site is located roughly 1200 base pairs (bp) downstream from the *PVT1* transcriptional start site (TSS), and 172 bp upstream of the miR-1204 stem-loop sequence. Therefore, the binding site is positioned in between exon 1A and exon 1B, and in very close proximity to the miR-1204 hairpin structure. The *PVT1* p53-response element is also conserved in mice, with only a slight variation of sequence and position

(not shown) Furthermore, an independent study which performed global analysis of p53-binding previously identified this region of the genome to be bound by p53 after 5-fluorouracil treatment in HCT116 cells (Wei et al., 2006)(<http://genome.ucsc.edu/>).

Using chromatin immunoprecipitation (ChIP) analysis, we found that this putative binding site was bound by endogenous p53. Furthermore, p53's binding to the *PVT1* p53-response element (*PVT1* RE) was enhanced by Daunorubicin treatment (Figure 2B). p53 bound the *PVT1* RE with an even greater affinity than another canonical p53 binding site, the p21 3' RE (as measured by primers located -1391 bp upstream of the p21 transcription start site). However, p53 bound the p21 5' RE (the stronger of the two well-established p53 REs in the p21 promoter) with two-fold greater affinity compared to the *PVT1* RE (data not shown). p53 was not found to significantly bind regions of *PVT1* where no consensus p53-binding sites had been identified: adjacent to the transcription start site (-398) or to an downstream exon (exon 8). An additional negative control 11 kilobase-pairs (kb) downstream of the p21 gene further confirmed the specificity of the p53-immunoprecipitation (Figure 2B).

To confirm that the ability of p53 to bind to the *PVT1* locus depended on the *PVT1* RE that we had identified, we used electrophoretic mobility shift assays (EMSA) (Figure 3). In order to perform this assay, we fluorescently labeled a 44-bp DNA fragment consisting of the *PVT1* RE (20 bp) flanked by the DNA sequence from the surrounding genomic context (Figure 3B). Using this probe, we assayed for the ability of purified Flag-tagged p53 to form a complex with the *PVT1* RE. Increasing amounts of purified p53 caused an increasing curve of up-shifted *PVT1* RE, indicative of direct binding by p53 to the *PVT1* RE. This up-shifted probe could also be super-shifted by

anti-Flag antibody, further confirming that this upshifted band represents the p53/*PVT1* RE complex (Figure 3A). To test our hypothesis that p53 binding of the *PVT1* RE required sequence specificity, we generated a *PVT1* mutant RE. This mutated *PVT1* RE was altered in only 4 nucleotide residues out of 20, the invariant G/Cs of the consensus p53-binding site (Figure 3B). We used excess amounts of either unlabeled wild-type *PVT1* RE or unlabeled mutant *PVT1* RE as potential binding competitors to the p53-*PVT1* RE complex. These assays revealed that an increasing amount of unlabeled wild-type probe is able to compete away the interaction between p53 and the labeled *PVT1* RE. However, an increasing amount of mutant *PVT1* RE was unable to compete for p53 binding (Figure 3C). Thus, the interaction between p53 and the *PVT1* RE requires sequence specificity.

p53 facilitates transactivation from the PVT1 Response Element

After establishing a direct interaction between p53 and the *PVT1* RE, we assayed for the ability of p53 to initiate transcription from this site. To do so, we cloned either the wild-type or mutant *PVT1* RE into luciferase-reporter constructs. These constructs were co-transfected with empty-vector or p53-containing plasmids into the p53-null H1299 lung-cancer cell line. As can be seen in Figure 4A, the addition of ectopic p53 caused a stimulation of transcription from the *PVT1* RE, thus resulting in an approximate 15-fold increase in luciferase activity. However, no increase in luciferase activity was detected in cells co-transfected with p53 and mutant *PVT1* RE, confirming that only the wild-type *PVT1* RE is competent for transcriptional activity mediated by p53.

We also assessed the ability of endogenous p53 to activate transcription that is dependent on the wild-type *PVT1* RE (Figure 4B). To do so, we transfected HCT116 or HCT116 p53^{-/-} cells with the luciferase constructs containing wild-type or mutant *PVT1* RE. Notably, transcription mediated by the wild-type *PVT1* RE generated ~5-fold greater luciferase activity in HCT116 cells compared to their p53 null counterparts (HCT116 p53^{-/-}). Despite a difference of only 4 nucleotides, the luciferase activity driven by the wild-type *PVT1* RE was ~24-fold higher than the mutant *PVT1* RE in HCT116 cells. This difference in steady-state luciferase activity is primarily due to the presence of p53, as only a ~2-fold difference in luciferase activity between wild-type and mutant *PVT1* REs was observed in HCT116 p53^{-/-} cells. Thus, the basal levels of endogenous p53 are sufficient to activate transcription from this response element in a sequence-specific manner.

Furthermore, stabilization of endogenous p53 levels by Nutlin treatment causes a greater than 2-fold increase in luciferase activity of the wild-type *PVT1* RE, but no increase in activity of the mutant *PVT1* RE. In addition, Nutlin-treatment of HCT116 p53^{-/-} cells did not result in up-regulation of luciferase activity driven by the wild-type *PVT1* RE (Figure 4B). Surprisingly, treatment of HCT116 cells with Daunorubicin failed to induce luciferase activity from the *PVT1* RE (data not shown).

p53 upregulates miR-1204

Given the observations that p53 both binds and stimulates transcription at the *PVT1* locus, we wished to explore the function of p53-mediated activation of this locus. While p53 induces the transcription of *PVT1* ncRNA (Figure 1), the *PVT1* locus also

encodes a cluster of microRNAs, miR-1204, miR-1205, miR-1206, miR-1207 5p, miR-1207 3p and miR-1208. As these microRNAs are likely to play biologically significant roles, we investigated the ability of p53 to induce their transcription. To do so, we designed qPCR primers that amplify DNA sequences corresponding to the primary microRNA molecules. Figure 5A demonstrates that the steady state levels of primary miR-1204, miR-1205, miR-1206 and miR-1207 are higher in HCT116 cells than in HCT116 p53^{-/-} cells.

Treatment of HCT116 cells with either Nutlin or Daunorubicin resulted in an increase of all the primary microRNAs of this locus with the exception of miR-1208 (Figure 5A). Notably, HCT116 p53^{-/-} cells failed to induce or only moderately induce primary microRNA levels. Interestingly, the different stimuli (Nutlin and Daunorubicin) used to activate p53 in HCT116 cells resulted in markedly different levels of primary microRNA induction. Yet, within the context of either treatment the fold-induction of the all primary microRNAs (except miR-1208) was similar, perhaps indicating that these primary microRNAs are transcribed in a polycistronic fashion. It remains possible, however, that these primary microRNAs arise from separate transcripts, and coincidentally are induced to similar levels. Regardless, miR-1208 seems to be regulated distinctly from the other microRNAs, as the levels of its primary transcript were not dependent on basal-levels of p53, and were repressed in a p53-independent manner following Daunorubicin treatment.

As microRNAs function as mature 19-22 nt species, we aimed to determine whether the transcriptional induction of the primary transcripts was sufficient to up-regulate mature microRNA levels. To do so, we employed a qPCR strategy specific for

mature microRNA detection (Qiagen miscript). Consistent with the induction of primary miR-1204, Daunorubicin treatment of HCT116 resulted in a ~7.5 fold-induction of mature miR-1204. In contrast, miR-1207-5p was not induced under these conditions (Figure 5B). Daunorubicin-treatment also caused the induction of the canonical p53-inducible microRNA miR-34a, but did not alter the levels of an unrelated small nucleolar RNA, SnoRD48 (data not shown).

Ectopic miR-1204 expression causes anti-proliferative phenotypes

Since levels of mature miR-1204 are increased by activation of p53, we investigated the function of miR-1204 as well as the other microRNAs of the *PVT1* locus. In order to do so, we transfected microRNA mimic molecules into various cancer cell lines, including HCT116 cells. In HCT116 cells, ectopic expression of miR-1204 causes a significant amount of cell death compared to cells transfected with negative control RNA. Notably, this level of cell death is comparable to that observed after ectopic expression of miR-34a. In addition, microRNA mimics for miR-1205, miR-1207-5p and miR-1207-3p also increased cell death. However, miR-1206 mimics did not cause cell death, nor did they alter the cell cycle profile of HCT116 cells (Figure 6A). Two additional observations strengthen the conclusion that miR-1204 specifically causes cell death of HCT116 cells: First, the overexpression an unrelated microRNA, miR-21, did not cause cell death, and second, the increase in cell death upon introduction of ectopic miR-1204 could be neutralized by a microRNA inhibitor of miR-1204, suggesting that miR-1204 kills cells via specific pathways, and not through toxic accumulation of the

RNA-based mimic (data not shown). Finally, we have observed that like miR-34a (Yamakuchi et al., 2008), ectopic miR-1204 expression stabilizes p53 levels (Figure 6B).

In other cancer cell lines, the ectopic expression of miR-1204 did not lead to cell death, but in fact resulted in cell cycle arrest at the G1 phase. For example, two diverse cancer cell lines, RKO (colon cancer) and Sk-Hep1 (liver cancer), were arrested in G1 after miR-1204 transfection (Figure 6C). In these settings, miR-1206 mimics again showed no phenotypic effect on the cell cycle profile. Finally, the overexpression of miR-1204 also decreased the viability of normal human fibroblast cells (WI38), suggesting that its anti-proliferative effects are not a consequence of interactions with cellular pathways that have become deregulated through the process of tumorigenesis (Figure 6D).

Discussion

We have characterized a novel p53-inducible target: the non-protein coding *PVT1* locus. We identified a canonical response element downstream of the *PVT1* transcription start site, adjacent to both Exon 1B and miR-1204. Purified p53 binds this site *in vitro* with sequence specificity, while endogenous p53 binds this site both in the basal state as well as upon p53 stabilization/activation by Daunorubicin-treatment. The presence of this site facilitates transcriptional activation mediated by p53, as luciferase constructs containing only a wild-type *PVT1* RE sequence are transactivated by the presence of p53 (either ectopic or endogenous). Furthermore, endogenous RNA species produced by this locus are induced by in a p53-dependent manner. These species include *PVT1* non-coding RNA (exon-containing), primary microRNA, and mature microRNA.

Our results revealed that mature miR-1204 is induced by DNA-damage. However, HCT116 colon cancer cells contain significantly lower levels of endogenous mature miR-1204 than miR-34a (data not shown). Therefore, a ~7.5 fold-induction represents a drastic change in the cellular levels of miR-1204 that may significantly impact cellular fate. As ectopic miR-1204 stabilizes p53 and causes cell death, basal levels of miR-1204 may be tightly regulated. Alternatively, miR-1204 may be expressed more highly in other cell lineages and exert its biological function within those settings. Future work will aim to determine the expression pattern of miR-1204 across multiple tissue types.

Delineating the extent of p53-dependent *PVT1* induction in response to different stimuli has already begun to provide insight into both *PVT1* regulation and function. Our data reveal that DNA-damage activated p53 more robustly upregulates *PVT1* ncRNA/primary microRNAs than stabilization of p53 in the absence of genotoxic stress (Nutlin-treatment). As DNA-damage cooperates with p53 to induce the *PVT1* locus, *PVT1*-encoded transcripts (microRNA or otherwise) may function in DNA-damage repair pathways, or in apoptosis-related pathways that initiate when resulting cellular insults have become too severe. Surprisingly, daunorubicin did not stimulate transcriptional activity from a luciferase construct containing the *PVT1* p53 RE. This suggests that additional factors that bind outside of the immediate region of the *PVT1* RE contribute to the p53-mediated activation of endogenous *PVT1*.

In light of our observations that implicate p53 in the control of the *PVT1* locus, several questions arise about the biological consequences of such regulation. First and foremost, why does p53 induce transcription from a seemingly oncogenic genomic locus?

As described in the introduction, the deregulation of this locus (through amplification and translocation of *PVT1*) is observed in a wide array of cancers. Furthermore, RNA interference of *PVT1* in cancer cell lines has resulted in increased cell death and enhanced sensitivity to chemotherapeutics. p53, on the other hand, is one of the most important cellular suppressors of tumor formation. Despite this, pro-survival roles for p53 have been previously described. For example, p53 is able to induce the transcription of death decoy receptors, *DcR1/TRID* and *DR2/TRUNDD* (Sheikh and Fornace, 2000). Such receptors compete with functional death receptors (like p53-inducible *KILLER/DR5* (Wu et al., 1997)) for ligand binding but do not transmit pro-apoptotic signaling, and thereby inhibit extrinsic apoptosis. Furthermore, p53 also blunts its ability to facilitate apoptosis by the intrinsic pathway as well. Here, p53 induces the transcription of *Slug*, a protein that suppresses transcription of the p53-inducible target, and potent pro-apoptotic gene, *PUMA* (Wu et al., 2005; Zilfou et al., 2005). Finally, p21, the first p53-target gene to be described, also plays anti-apoptotic roles (Janicke et al., 2007).

Thus, it is not without precedence that p53 induces genes that counteract cell death, and all indications to date suggest that the *PVT1* gene would fall under this category. However, the mechanism by which *PVT1* opposes cell death has not been explored. As previously mentioned, many non-coding RNAs interact with and direct gene silencing by the polycomb repressive complex (Zhao et al., 2010). Further, recent work has suggested that the deregulation of ncRNA-directed gene silencing contributes to tumorigenesis (Gieni and Hendzel, 2009). As the *PVT1* locus is often deregulated in tumors, *PVT1* ncRNA could potentially direct polycomb group gene silencing, and contribute to aberrant gene silencing.

Due to the close proximity of the *PVT1* and *c-Myc* loci, gross alterations of *PVT1* are thought to affect *c-Myc* expression (Beck-Engeser et al., 2008). For example, translocations of *PVT1* observed in mouse plasmacytoma are often accompanied by transcriptional changes within the *c-Myc* locus (Lazo et al., 1990; Marcu et al., 1992). Further, co-mutational analysis has suggested that *PVT1* and *c-Myc* operate within the same oncogenic pathway (Beck-Engeser et al., 2008). While such evidence points toward cooperation between *PVT1* and *c-Myc*, the observations that ncRNAs often repress transcription warrants further exploration into the functional and mechanistic interface between *PVT1* ncRNA and *c-Myc* expression. As p53 is known to repress *c-Myc* through multiple mechanisms (Ho et al., 2005; Sachdeva et al., 2009), future studies using antisense *PVT1* RNA will aim to determine whether p53-dependent induction of *PVT1* ncRNA positively or negatively regulates *c-Myc* expression. Examining the consequences of *PVT1* ncRNA induction on *c-Myc* expression (as well as genome-wide mRNA expression changes) will begin to elucidate the mechanism by which *PVT1* promotes cell survival.

As the experiments linking *PVT1* with cell survival used RNA interference targeting exonic regions of *PVT1* ncRNA, it is uncertain (and perhaps unlikely) that such strategies inhibited *PVT1*-encoded microRNAs. In contrast to a pro-survival role for *PVT1*, our data suggests that miR-1204 plays an anti-proliferative role, inducing apoptosis or cell cycle arrest. These phenotypes are more in line with the well-established roles of p53, and as such miR-1204 may represent a more obvious functional target of p53 at the *PVT1* locus. Supporting this hypothesis is the location of the p53 binding site, which is in close proximity to both Exon 1B and miR-1204 (a mere 172-bp

away from the annotated stem-loop precursor of miR-1204). The binding of p53 to this site could potentially explain the marked p53-dependent induction of both Exon 1B and mature miR-1204. That is, transcription from this specific genomic region would be enhanced by p53 binding with corollary up-regulation of miR-1204. Perhaps p53-binding results in the usage of an alternative transcription start site directly upstream of Exon 1B- future studies will address this possibility.

Alternatively, p53 may regulate the increase in mature miR-1204 levels by directing microRNA biogenesis events. As microRNA processing is heavily regulated, the levels of primary microRNA transcripts do not necessarily dictate levels of mature microRNA. In fact, our data demonstrate that the induction of primiR-1207 is also induced by p53, but without induction of the mature miR-1207-5p. This could be explained by preferential incorporation of miR-1207-3p into the RISC complex, with subsequent degradation of miR-1207-5p. Experiments to address this possibility are ongoing. If mature miR-1207-3p levels are not induced, however, this would suggest that the processing efficiency of miR-1204 is greater than that for miR-1207. Intriguingly, p53 has been linked to processing efficiency of microRNAs through an interaction with Drosha (Suzuki et al., 2009), and thus it is tempting to speculate that p53 binding to the genomic region adjacent to miR-1204 somehow facilitates co-transcriptionally-dependent microRNA processing events.

If miR-1204 represents a functional target of p53 at the *PVT1* locus, the additional ncRNA transcripts (those that include exonic *PVT1* sequences) may represent either transcriptional by-products of miR-1204 regulation, or may represent additional functional targets. If the latter scenario is correct, activation of the *PVT1* locus may

represent a double-edged sword- that is, induction of miR-1204 may promote apoptosis (or arrest), while the *PVT1* ncRNAs may prevent apoptosis. Thus, p53 may exert control over the ratio of such transcripts to fine tune cellular responses to certain stimuli. In this way, the *PVT1* locus could be viewed as a molecular switch between life and death.

While still very much speculative, cancer cells may have manipulated the balance of such transcripts through deregulation of the locus, producing more *PVT1* pro-survival ncRNAs than anti-proliferative miR-1204 (and perhaps the other microRNAs of this locus).

Future experiments will begin to test this hypothesis in order to understand the precise interplay between the microRNAs and other ncRNAs of the *PVT1* locus, and ultimately how they function within the context of the p53-pathway.

Materials and Methods:

Cell culture and transfections: HCT116 (colorectal carcinoma), HCT116 p53^{-/-}, RKO (colorectal carcinoma), Sk-Hep1 (hepatocellular carcinoma) and WI-38 (normal human fibroblasts) cells were maintained in DMEM/10% FBS. HCT116 wild type cells and their derivatives HCT116 p53^{-/-} were generously provided by B. Vogelstein. HCT116 and HCT116 p53^{-/-} (colorectal carcinoma) cells were treated with Daunorubicin (0.22 μ M; Sigma) or Nutlin-3 (10 μ M; Sigma-Aldrich) for 24 hours unless otherwise noted.

microRNA mimics were purchased from Qiagen and used individually at 20 nmol unless otherwise noted, and transfected into cells using Dharmafect 1 reagent (Dharmacon) according to the manufacturer's instructions. Control microRNA (Qiagen All-Stars) represents a negative control microRNA mimic.

Western Blot Analysis: Cell extracts were analyzed according to standard Western blotting procedures using fluorescence through use of the Odyssey system (LI-COR).

The monoclonal antibodies DO1 or 1801 were used to detect p53. The following polyclonal antibodies were purchased from either Santa Cruz, sc-502, anti-p21 (C-19), or Sigma: Anti-Actin. (A2066).

QRT-PCR to assess changes in the expression of PVT1 transcripts: Quantitative Reverse Transcription Polymerase Chain Reaction was used to quantify relative changes in *PVT1* ncRNA and primary microRNA expression. RNA was isolated from cell cultures using the Qiagen RNeasy Mini Kit. cDNA was synthesized by the use of the Qiagen Quantitect Reverse Transcription Kit. PCR was carried out on an Applied Biosystems Step One Plus using the SYBR Green dye (Applied Biosystems). RNA expression was assayed in triplicate and normalized to RPL32 mRNA expression. Relative levels were calculated by the Comparative-Ct Method ($\Delta\Delta C_T$ Method). Error bars are derived from the standard deviations of multiple experiments.

Primers were designed with *Primer Express* (Applied Biosystems):

RPL32 F: TTCCTGGTCCACAACGTCAAG

RPL32 R: TGTGAGCGATCTCGGCAC

primiR-1204 F: GGCACAAGGGCCCAACT

primiR-1204 R: TCCCTCTGGGAATTCAATGG

primiR-1205 F: CCTTCCTGTCAACCCTGTTCTG

primiR-1205 R: CAGTTATCTATGCCTCAGTTTTTGTGT

primiR-1206 F: CAGTGTTTCATGTAGATGTTTAAGCTCTTG

primiR-1206 R: GCATAATTTGGCAGCGTTCA

primiR-1207 F: GCTGGCTGGGTCTGGTAGTG

primiR-1207 R: GGTCAGCTAACAGAAGTGCTGTCTT

primiR-1208 F: TGGTGGGCAACAATGAATCA

primiR-1208 R: CCGTCTCCGCCTGTCTGA

Exon 1A F: CGGCACCTTCCAGTGGAT

Exon 1A R: CCGTGTCTCCACAGGTCACA

Exon 1B F: TCCCGGAAGCTGCAGAAG

Exon 1B R: CCAGGCCACGAGGTTTCTC

Exon 4 F: TGGCACATACAGCCATCATGA

Exon 4 R : ACGTGCCAAGCAGCTCAAA

QRT-PCR to assess expression changes of mature microRNAs:

HCT116 cells were plated to ~50% confluency (24 hours) in 6 well plates with at least 3 replicates per sample. At 24 hours, cells were treated with 0.22 μ M Daunorubicin (Sigma) or untreated to a total volume of 5ml of RPMI1640 plus 10% HBS. After addition of Daunorubicin, cells were harvested for RNA at 24 hours using the mirVana miRNA isolation protocol (Ambion #1560). Mature miRNA was assayed by synthesis of cDNA using the miScript PCR system protocol (Qiagen) at 20 μ l final volume (37°C, one hour). miRNA specific real time fluorescence amplification was carried out with 5' gene specific primers and a 3' universal primer annealing to the synthetic oligo dT sequence (Denaturation 15s, 94°C, Annealing 30s, 55°C, Extension 30s, 70°C) for 35 cycles. Datapoints used are the average of 3-4 values each and are normalized to Hs_RNU6B_3 (Qiagen MS00029204). Gene specific primers were obtained from Qiagen and are the following: Hs_miR-1204_1 (MS00014161) and Hs_miR-1207-5p_1 (MS00014189).

Flow Cytometry Analysis: Cell pellets were washed with PBS and fixed/permeabilized with 50% ice-cold ethanol. Pellets were washed and resuspended in 50 μ g/ml Ribonuclease A and 62.5 μ g/ml propidium iodide. Samples were analyzed on the FACSCalibur Becton Dickinson. The percentages of cells in various phases of the cell cycle were quantified using the ModFit *LT Version 3.0* program. Error bars are derived from the standard deviations of multiple experiments. Levels of significance were

calculated using the student t-test. Statistical significance ($p < 0.05$) is denoted with an asterisk.

Chromatin Immunoprecipitation Analysis:

ChIP analysis was carried out as previously described (Beckerman et al., 2009). Briefly, p53 was immunoprecipitated with monoclonal antibodies DO1 and 1801. A no-antibody control was also included to confirm the specificity of the immunoprecipitation.

ChIP DNA was isolated with the Qiaquick PCR Purification Kit (Qiagen). Subsequent qPCR using 1/50th fraction of ChIP-enriched DNA, and 100 nM primers in a total volume of 20 μ L was conducted to assess the amount of DNA that had been precipitated. Standard curves from 0.1–100 ng of sonicated genomic DNA were also amplified by qPCR as a reference. The following primers were used for qPCR:

PVT1 RE F: TGCATACTGGCAGCGACAAG
PVT1 RE R: TTCGCTATGACCACAGGACTGT
PVT1 -398 F: GGTAAGAGGGCTCAGGGAAAGA
PVT1 -398 R: GTGATGCCAGCTCGCTTGT
PVT1 Exon 8 F: CGGGCTCCCAGATTCACA
PVT1 Exon 8 R: TCAGCCTCCAAGCGTTCCT
p21 3'RE F: CTGTCCTCCCCGAGGTCA
p21 3' RE R: ACATCTCAGGCTGCTCAGAGTCT
p21 +11kb F: TCTGTCTCGGCAGCTGACAT
p21 +11kb R: ACCACAAAAGATCAAGGTGAGTGA

Luciferase Assays:

The following 70 nucleotide oligonucleotides were purchased from IDT, annealed and cloned into the KpnI-XhoI sites of the luciferase reporter PGL2 (Promega)

Wild-type PVT1 RE Top Strand:

CTCAGACCTATTTTGCATACTGGCAGCGACAAGTTGAGACTTGTTCAACTTGA
CACAGTCCTGTGGTCATAC

Wild-type PVT1 RE Bottom Strand:

TCGAGTATGACCACAGGACTGTGTCAAGTTGAACAAGTCTCAACTTGTCGCT
GCCAGTATGCAAAATAGGTCTGAGGTAC

Mutant PVT1 RE Top Strand:

CTCAGACCTATTTTGCATACTGGCAGCGAAAAATTGAGAATTATTCAACTTGA
CACAGTCCTGTGGTCATAC

Mutant PVT1 RE Bottom Strand:

TCGAGTATGACCACAGGACTGTGTCAAGTTGAATAATTCTCAATTTTTCGCTG
CCAGTATGCAAAATAGGTCTGAGGTAC

Cells were transfected such constructs using Lipofectamine 2000 (Invitrogen). Renilla was co-transfected for the purposes of normalization. Luminescence was analyzed on the 20/20ⁿ luminometer (Turner biosystems)

Electrophoretic Mobility Shift Assay (EMSA):

The following DNA oligonucleotides were purchased from IDT and used to generate EMSA probes. The top and bottom strands were annealed to create double-stranded DNA. *PVT1* PE labeled wild-type probes were labeled with IRDye800 at the 5' end of both top and bottom DNA strands. The region in bold denotes the p53-response element (*PVT1* RE). Italicized nucleotides indicate mutations introduced in the invariant region of the *PVT1* RE.

Wild-type *PVT1* RE: 44 bp

Top strand: GCATACTGGCAG**CGACAAGTTGAGACTT**GTTCAACTTGACACAG
Bottom strand: CTGTGTCAAGTTGAACAAGTCTCAACTTGTCGCTGCCAGTATGC

Mutant *PVT1* RE: 44 bp

Top: GCATACTGGCAG**CGAAAAATTGAGAATTATT**CAACTTGACACAG
Bottom: CTGTGTCAAGTTGAATAATTCTCAATTTTTCGCTGCCAGTATGC

Purified Flag-tagged p53 (0 ng-50 ng) was incubated with 10 ng labeled wild-type *PVT1* RE probe. Reactions were run out onto 4% native acrylamide gels to assess the formation of p53-DNA complexes (upshifted products). The labeled probe was

visualized with fluorescence detection through use of the Odyssey system (LI-COR). In competition assays, unlabeled competitor probe was added in a range of 0-250 ng.

Cell Viability Assays:

WI-38 cells were transfected with control or miR-1204 mimic or treated with DMSO/Nutlin. 72 hours post-transfection, cells were stained with Crystal Violet solution. Absorbance at 590 nm was measured by a Victor3 plate reader (PerkinElmer). Error bars are derived from the standard deviations of multiple experiments. Levels of significance were calculated using the student t-test. Statistical significance ($p < 0.05$) is denoted with an asterisk.

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Conflict of Interest:

The authors declare no conflict of interest.

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Figure Legends:

Figure 1. p53 stimulates transcription at the *PVT1* locus. **A.** HCT116 or HCT116 p53^{-/-} cells were treated with either Nutlin-3a (10 μ M) or Daunorubicin (0.22 μ M). Levels of p53, p21 and actin (loading control) were detected by immunoblotting **B.** Quantification of *PVT1* ncRNA by qPCR of HCT116 cells (hatched bars) and HCT116 p53^{-/-} cells (black bars) as treated in (A.) **C.** Quantification of *PVT1* alternative exons 1A (hatched bars) and 1B (black bars) by qPCR in HCT116 and HCT116 p53^{-/-} cells treated as in (A).

Figure 2. Endogenous p53 binds to a canonical response element within the *PVT1* locus. **A.** Schematic diagram of the p53 RE in relation to other points along the *PVT1* locus. Gray nucleotides represent deviations from the canonical p53 response element. The locations of primer pairs used in ChIP analysis are denoted. **B.** HCT116 cells were untreated (hatched bars) or treated 0.22 μ M Daunorubicin (black bars) and subjected to Chromatin Immunoprecipitation (ChIP). qPCR was used to quantify DNA fragments co-immunoprecipitated with p53, or with beads alone (gray bars). Three primer pairs within the *PVT1* locus were used to assay for p53 binding, -398, *PVT1* RE, and Exon 8. Two additional primer pairs at the p21 locus, the 3' p53 RE (-1391), and +11 kb (downstream of the p21 gene), were used as positive and negative controls, respectively. The extent of relative p53-binding to each genomic region is graphed.

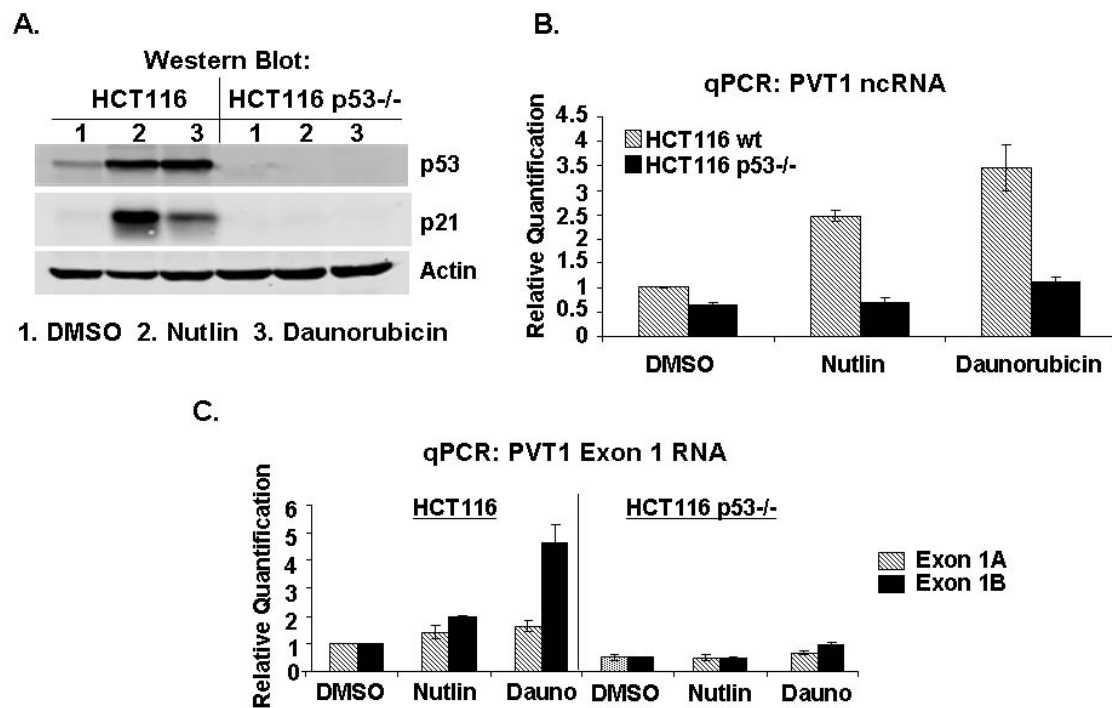
Figure 3. p53 directly binds the *PVT1* RE in a sequence specific manner. **A.** EMSA assay: an increasing amount of purified, flag-tagged p53 was incubated with labeled *PVT1* RE probes. Such reactions were loaded onto a 4% native polyacrylamide gels. Only the upshifted probe is displayed. Flag antibody was added into one reaction in order to supershift this complex. **B.** The sequences of both wild-type and mutant *PVT1* RE. Gray nucleotides indicate mutations of the probe at invariant residues of the p53-consensus sequence. **C.** Competition EMSA: Excess unlabelled *PVT1* RE (either wild-type or mutant) probe were added in increasing amounts to reactions described in (A).

Figure 4. p53 facilitates transcriptional activity from the *PVT1* RE. Reporter constructs were engineered to contain either wild-type or mutant *PVT1* RE that drive the transcription of the Firefly *luciferase* gene. **A.** Empty vector or ectopic p53 were co-transfected with the luciferase reporter constructs in p53-null H1299 cells. Relative luminescence is quantified: empty vector (hatched bars), p53 (black bars). **B.** HCT116 (hatched bars) or HCT116 p53^{-/-} cells (black bars) were transfected with either wild-type or mutant *PVT1* RE containing-luciferase constructs. Relative luminescence is quantified.

Figure 5. p53 induces primary and mature miR-1204. **A.** HCT116 or HCT116 p53^{-/-} cells were treated with either Nutlin-3a (10 μ M) or Daunorubicin (0.22 μ M). Relative levels of the primary microRNA transcripts were quantified by qPCR: The relative quantification of primiR-1204 (hatched bars), primiR-1205 (black bars with white dots), primiR-1206 (horizontal striped bars), primiR-1207 (gray bars) and primiR-1208 (black

bars) is displayed. **B.** HCT116 cells were treated with 0.22 μ M Daunorubicin. The microRNA enriched RNA fraction was subjected to Qiagen miscript cDNA synthesis and qPCR. Relative quantification of mature miR-1204 and miR-1207-5p is displayed and represents the fold induction of each RNA species after Daunorubicin treatment. **C.** Schematic of the *PVT1* locus located at human chromosome 8q24. Black boxes denote exons or alternative exons of *PVT1*. The position of the microRNA stem-loop structures are denoted by gray boxes. This schematic is adapted from Huppi et al (2008) (Huppi et al., 2008).

Figure 6. Ectopic miR-1204 induces apoptosis or cell cycle arrest. **A.** HCT116 cells were transfected with 20 nmol microRNA mimics. Cells were subjected to propidium iodide staining and FACs analysis. The cell cycle profile following transfection of each mimic is shown. The percentage cell death, as quantified by Sub-G1 content, is graphed. **B.** HCT116 cells were transfected as in (A). The levels of p53 and actin (loading control) were detected by immunoblotting. **C.** RKO or Sk-Hep1 cells were transfected and subjected to FACs analysis as in (A). The percentages of cells in G1, G2/M or S-phase is quantified. **D.** WI-38 normal human fibroblasts were transfected as in (A). Cell viability was measured by Crystal Violet assays. In (A)-(D), levels of significance are denoted by asterisks.

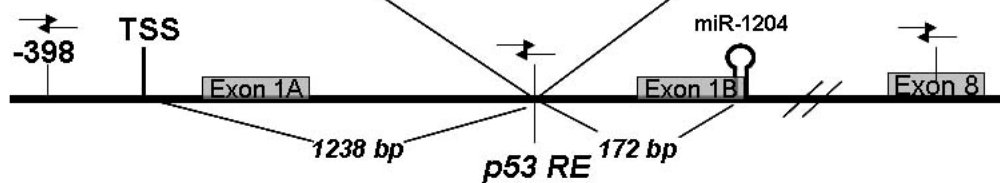


Barsotti et al Figure 1

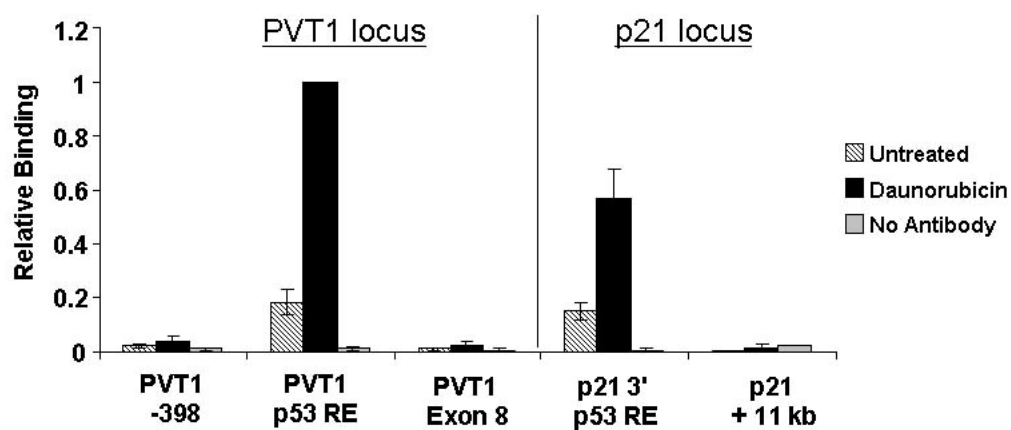
A.

PVT1 locus: PVT1 p53 RE

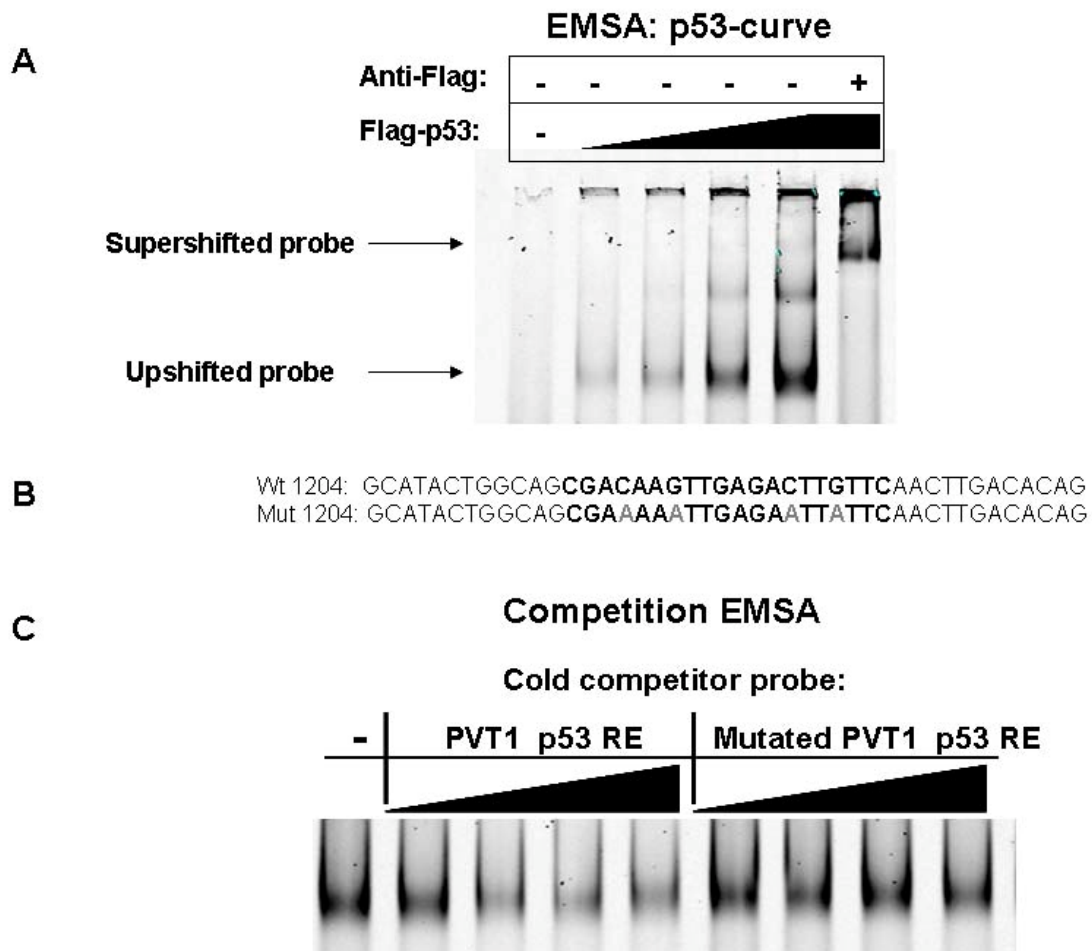
p53 consensus binding site: RRRCWWGYYY . RRRCWWGYYY
 CGACAAGTTG . AGACTTG TTC



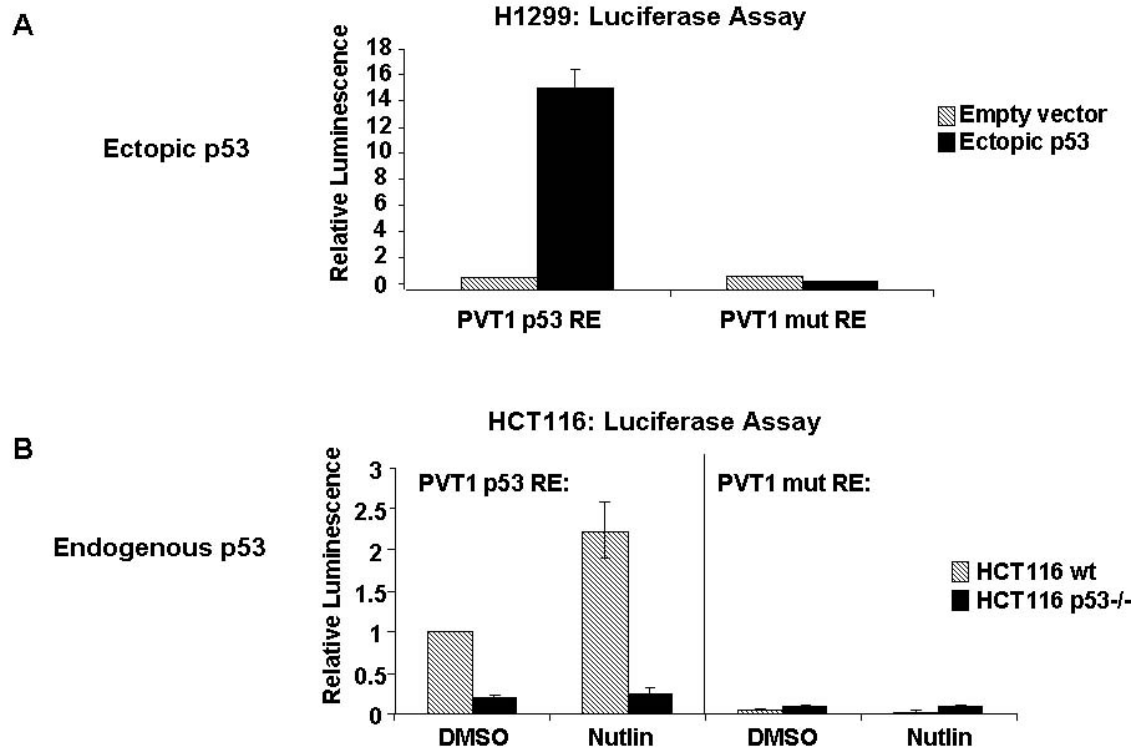
B.

HCT116 p53 ChIP:

Barsotti et al Figure 2

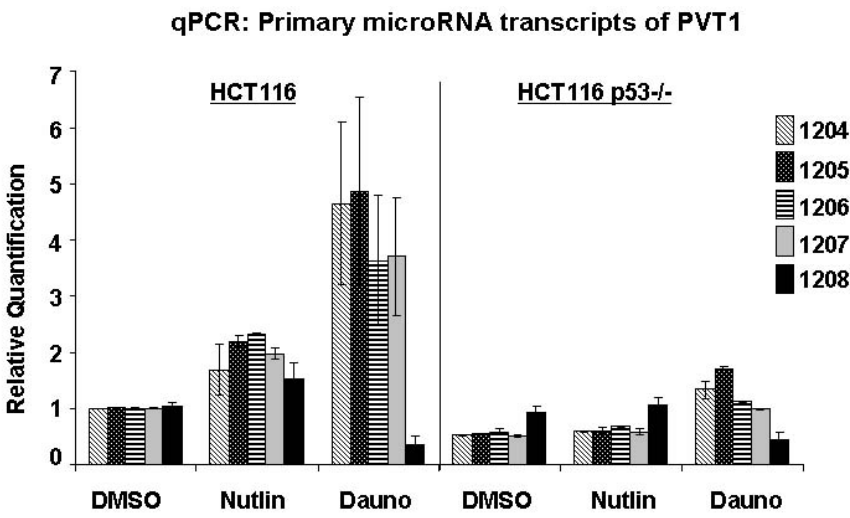


Barsotti et al Figure 3

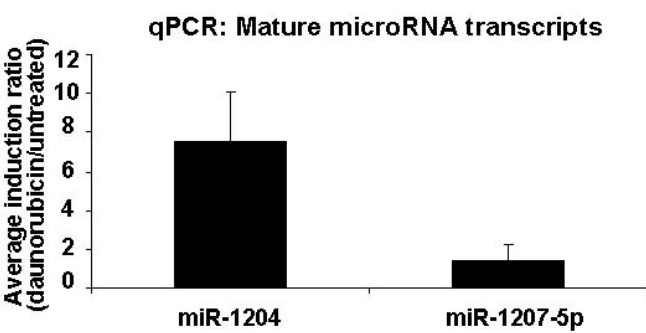


Barsotti et al Figure 4

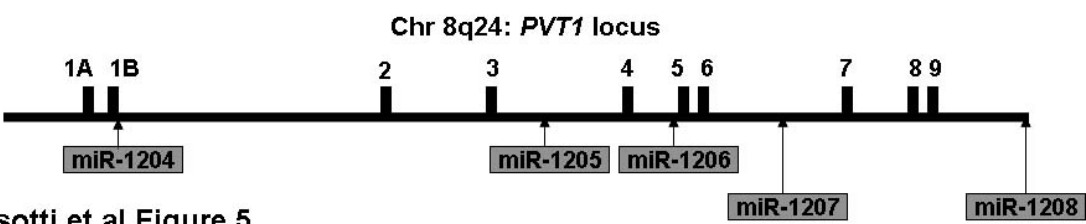
A.



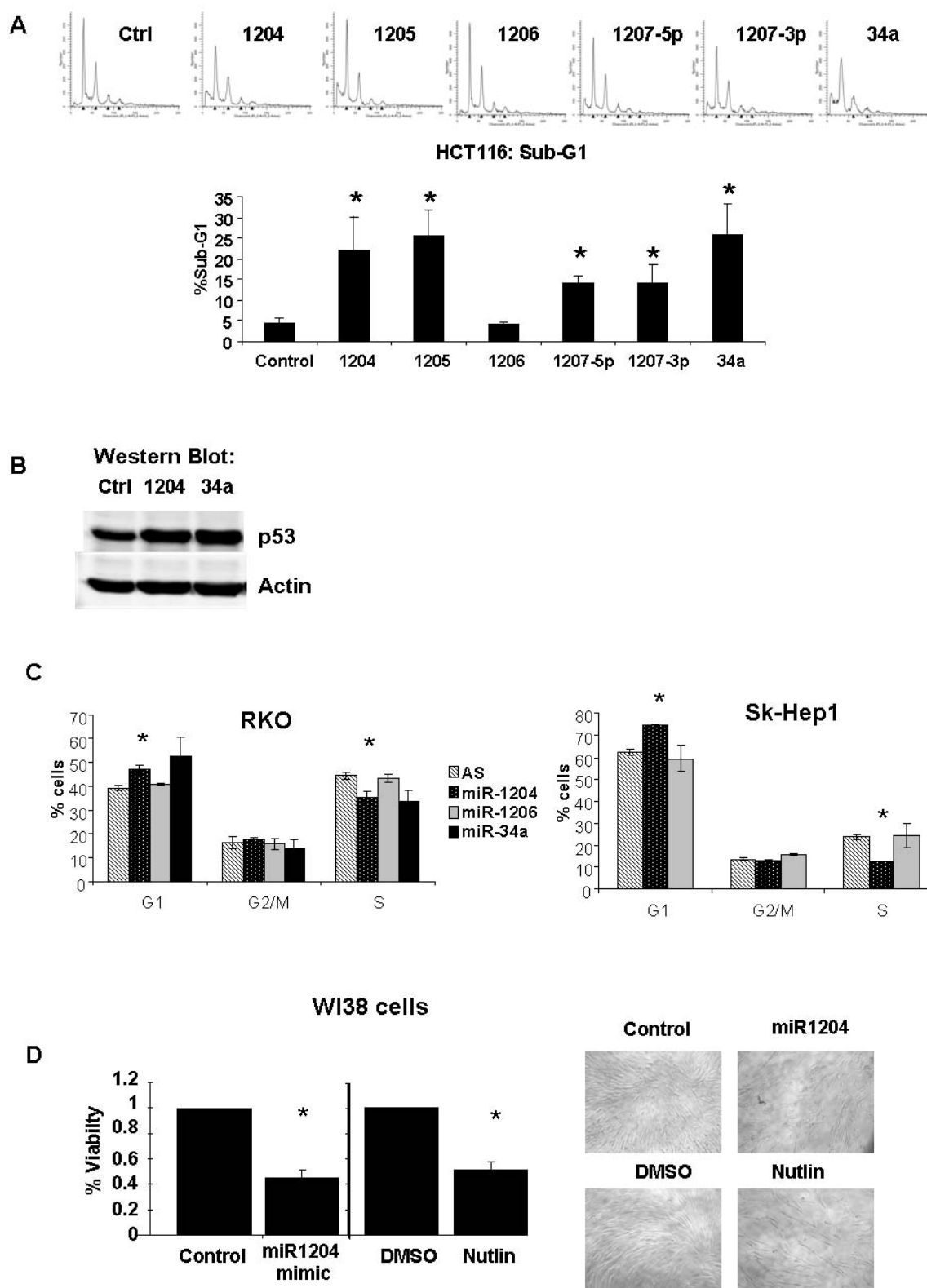
B.



C.



Barsotti et al Figure 5



Barsotti et al Figure 6

Chapter 4

Perspectives and future directions

p53 is the one of the most, if not the most, frequently mutated genes in human cancer. Even when spared from mutation, activity of the p53 pathway is often suppressed in cancer cells by other means. For example, p53 is targeted for inactivation by several tumor virus-encoded proteins, and is also inhibited by the overexpression of its negative regulators, Mdm2 and Mdmx. Furthermore, Mendelian genetics have revealed the importance of p53 to cancer biology as germline mutations in the *TP53* gene result in Li-Fraumeni syndrome, a genetically inherited disease that predisposes patients to very early onset of cancer. In addition to these clinical observations, a multitude of studies have delineated the importance of p53 to tumor suppression both *in vivo* and *in vitro*. In light of such observations, it is clearly advantageous to study the functions of p53 in the hopes that these functions can be restored in cancer cells. Accordingly, a massive body of literature has revealed a host of p53 functions that may contribute to p53's tumor suppressive ability, and such studies have indeed provided insight into possible chemotherapeutic intervention strategies.

Unfortunately, the task of therapeutically targeting a tumor suppressor is a formidable challenge. That is, simply put, because tumor suppressors are by definition "lost" during cancer formation and progression, and it is quite difficult to target something that (for argument's sake) just isn't there. On the other hand, p53 is not usually "lost" in cancer, but is most often inactivated by point mutations. Thus, the task is difficult- but maybe not impossible. Considerable effort has gone into developing molecules that may restore wild-type p53 function to the mutated versions of p53 found in many cancer cells. For example, a drug called PRIMA-1 has shown great success in

this realm, and as such there is room for cautious optimism (Lambert et al., 2009; Wiman, 2010). Furthermore, while p53 is frequently mutated in cancers, a subset of tumors do retain wild-type p53. Treatment of such cancer cells with drugs that inhibit Mdm2, like Nutlin-3a, give rise to marked upregulation of wild-type p53 levels and activity, and thus restoration of p53 function to tumor cells (Vassilev, 2005; Vassilev et al., 2004).

With these notable exceptions, insights into chemotherapeutic strategies stemming from the p53 field are likely to be derived through revelation of p53-dependent functions and pathways. Specifically, the elucidation of p53-regulated target genes may provide novel drug targets. Despite the tremendous importance of p53-inducible targets to p53-function (e.g. p21 for cell cycle arrest, PUMA for apoptosis), it is inherently difficult to restore the expression of such genes in cancer cells that contain inactivated versions of p53. (However, various scenarios to do so can be envisioned: regulation of protein/mRNA stability through inhibition of kinases/E3 ligases, epigenetic modifiers, gene therapy). On the other hand, one function of p53 that is particularly amenable to chemotherapeutic intervention is p53-mediated repression.

The study of p53-mediated repression often reveals genes that oppose p53 function, and on the whole, promote cell proliferation/block cell death and are thus capable of contributing to tumorigenic processes. Fortunately, these repression targets of p53 may represent viable cancer drug targets. When p53-activity is suppressed, these genes are deregulated, and often overexpressed. Furthermore, inhibition of such factors might mimic a true function of p53, and remains possible even in the absence of functional p53.

As previously mentioned, p53 serves to repress a substantial number of genes. However, neither a consensus mechanism, nor comprehensive set of commonly downregulated-genes has been established. Nonetheless, a large list of p53-repressed genes can be compiled, with a large proportion of such genes having roles in cell cycle proliferation and survival. However, other p53-repressed genes function in diverse biological processes, such as the maintenance of pluripotency or control of metabolic flux, which are processes that are becoming increasingly recognized as major contributors to carcinogenesis.

FoxM1

As cell cycle arrest is one of the central mechanisms regulated by p53, it is perhaps not entirely surprising that p53 represses the transcription of many genes that promote cell cycle proliferation. However, as p53-inducible targets clearly play important roles in halting cell growth, the significance of the repression of such genes is called into question. As many genes that function to regulate cell cycle events are themselves regulated by cell cycle progression, one plausible scenario is that the cell cycle block imposed by p53 leads to apparent gene repression. That is, the cell cycle may have simply been arrested at a point in the cell cycle when the expression of these genes is inherently low. Arguing against this hypothesis, however, are the observations that many p53-repressed genes are down-regulated in a timeframe that precede perturbations of the cell cycle profile (Spurgers et al., 2006). Regardless of the contribution of such genes to the induction of cell cycle arrest, the continuous repression of their expression would be expected to contribute to maintenance of a stable arrest. And in fact, this is

exactly in line with the role of p53, as many cell cycle checkpoints operate independently of p53 and with faster kinetics than p53-mediated arrest (Giono and Manfredi, 2006).

p53, then, is truly important to maintain cell cycle arrest. And it is within this context that p53-mediated repression is crucial: the overexpression of the p53-repressed genes c-Myc or Cyclin B1 overcome p53's ability to maintain both G1 or G2 arrest, respectively (Ho et al., 2005; Innocente et al., 1999).

The quantity and importance of p53-repressed cell cycle genes is quite striking. For example, p53 mediates the repression of many important regulators of the G2/M transition and mitotic progression: p53 inhibits the expression of both Cyclin B1 and Cdc2 (which together represent the essential Cdk complex that stimulates mitotic entry), as well as Cdc25C (the phosphatase that is required for the activation of that Cdk complex). Furthermore, p53 represses mitotic kinases (e.g. Plk1, Nek2, Aurora Kinase B (McKenzie et al., 2010; Spurgers et al., 2006)), regulators of microtubule dynamics (e.g. Stathmin (Murphy et al., 1999)), factors that interact with the centrosome-kinetochore complex (e.g. CENPF (Kumamoto et al., 2008; Spurgers et al., 2006)) and even components of ubiquitin ligase complexes that control cell cycle transitions (e.g. Cdc20 and Skp2 (Kumamoto et al., 2008; Spurgers et al., 2006)). While many elegant studies have established mechanisms of p53-dependent repression for several genes listed above, the down-regulation of a master transcriptional regulator of such genes by p53 would represent a comprehensive strategy to ensure the repression of such targets, and thereby enforce maintenance of cell cycle arrest. Our work has identified one such master regulator, FoxM1, as a target of p53-mediated repression (Barsotti and Prives, 2009). And in fact, FoxM1 has been reported to serve as an upstream transactivator of every

target gene listed above (Kalin et al., 2011; Laoukili et al., 2007; Wang et al., 2010; Zhang et al., 2006).

In Chapter 2, we described in detail the cellular conditions, mechanism and consequences of p53-dependent repression of FoxM1. Additional data that was not included in Chapter 2 has verified our conclusion that the repression of FoxM1 by p53 operates at the transcriptional level. To demonstrate that p53 represses transcription through the FoxM1 promoter, we employed luciferase assays using a reporter construct the transcription of which is driven by a fragment of the FoxM1 promoter (kindly provided by Eric Lam, (Kwok et al., 2008)). Both ectopic p53 transfected into p53-null H1299 cells, as well as Nutlin-activated endogenous p53 of MCF7 or HCT116 cells repressed FoxM1 promoter-driven luciferase. HCT116 p53^{-/-} cells, in contrast, did not inhibit transcription from this construct upon Nutlin-treatment (Figure 1). Furthermore, several studies have provided additional data that have validated our novel findings regarding the p53-dependent repression of FoxM1 (Alvarez-Fernandez et al., 2010; Millour et al., 2011; Pandit et al., 2009). In Chapter 2, we presented data suggesting that the transcriptional downregulation of FoxM1 involves activation of the Rb-family with subsequent inhibition of E2F1 (Barsotti and Prives, 2009). One recent study has now confirmed and extended these findings by demonstrating p53-dependent transcriptional repression of FoxM1 through an increase of Rb and decrease of E2F1 at the FoxM1 promoter (Millour et al., 2011).

Our data suggest an important role for p53-dependent repression of FoxM1 in the maintenance of a stable G2 arrest. Owing to the functional importance of this repression, our data reveals that FoxM1 repression by p53 is not simply a consequence of

cell cycle arrest at a point when FoxM1 levels are inherently low. FoxM1 levels are lowest in G1 phase, rise during late S-phase and peak in G2 (Laoukili et al., 2008; Laoukili et al., 2007; Park et al., 2008). In MCF7 cells, Daunorubicin treatment causes an accumulation of cells in G2 (high FoxM1 levels), but a decrease in cells in the G1 phase (low FoxM1 levels) (data not shown). Despite this, treatment of MCF7 cells with daunorubicin causes robust p53-dependent repression of FoxM1 (even when the majority of cells are in the G2 phase, as shown in Chapter 2). Furthermore, Nutlin-treated HCT116 cells represent a similar situation: Nutlin treatment induces accumulation of cells in G2 phase, but a drop in G1 phase. p53 activation by Nutlin causes a dramatic reduction in FoxM1 levels. Furthermore, our preliminary data demonstrate that synchronization of HCT116 cells in late G1/early S-phase (low FoxM1 levels) with L-mimosine does not down-regulate FoxM1 levels to an extent comparable to the repression observed after Nutlin treatment. This suggests a true and specific effect of p53 on FoxM1 expression that is independent of cell cycle stage (Figure 2).

As shown in Chapter 2, artificial down-regulation of FoxM1 levels using siRNA in cells that lack p53 expression rescues the ability of such cells to maintain stable G2 arrest in response to DNA-damage. Thus, robust p53-dependent FoxM1 repression is likely to be sufficient for maintenance of a stable G2 arrest- but is it necessary? This is an especially important question in light of the observation that a high percentage of cancer types overexpress FoxM1. Does such FoxM1 overexpression give tumor cells an advantage by allowing passage through G2 into mitosis? As many p53-dependent mechanisms have been implicated in the control of the G2/M transition, future work will attempt to address the precise contribution of FoxM1 repression to G2 arrest.

It has been previously shown that the overexpression of cyclin B1 abrogates p53-dependent G2 arrest (Innocente et al., 1999). As FoxM1 is required for timely expression of cyclin B1 (as well as many other G2/M specific genes) (Laoukili et al., 2005; Leung et al., 2001), our future studies will address whether or not FoxM1 overexpression counteracts p53-dependent G2 arrest. Preliminary experiments in a FoxM1-inducible system (U2OS C3 cells, kindly provided by I-Ching Wang, (Kalinichenko et al., 2004)) have attempted to address this question.

To assess the phenotype of ectopic FoxM1 expression on cells arrested in G2, we pre-treated U2OS C3 cells with or without tetracycline, and then treated these cells with Nutlin for ~3 days. Much like overexpression of Cyclin B1 (Innocente et al., 1999), ectopic FoxM1 expression decreases the percentage of cells that arrest in G2 in response to Nutlin and instead increases the amount of cells arrested in G1 (Figure 3). These results are in line with our hypothesis that ectopic FoxM1 may allow cells to more rapidly enter mitosis, partially overcoming the G2 arrest imposed by Nutlin-activated p53. However, as we have not addressed the precise mechanism by which ectopic FoxM1 causes a decrease in %G2 and accumulation of cells in G1 phase, these data must be interpreted with caution. An equally plausible scenario is that FoxM1 overexpression causes the activation of a cell cycle checkpoint at the G1/S transition. Future studies will discern the mechanisms that lead to accumulation in G1 phase and drop in %G2, as well as extend these findings into other cellular systems.

Furthermore, constitutive overexpression of FoxM1 will begin to shed light on p53's specific role in mitotic progression. While most cells remain stably arrested at G2 after FoxM1 repression, what happens to the population of cells that does enter mitosis?

Namely, does p53-dependent FoxM1 repression contribute to, or abrogate the spindle checkpoint (leading to deregulated mitosis)? Would prolonged repression of FoxM1 by p53 give rise to polyploidy (as observed in cardiomyocytes of FoxM1 null mice), or mitotic catastrophe (as observed in cell culture)? Alternatively, p53-mediated repression may in fact protect cells against such processes, thereby contributing to the maintenance of genomic stability. This may in fact be an important ramification of FoxM1 repression, as FoxM1 overexpression has been shown to contribute to genomic instability (Gemenetzidis et al., 2009). The full extent of mitotic phenotypes associated with p53-dependent repression of FoxM1 awaits further characterization.

In addition to its prominent role in promoting cell cycle progression, FoxM1 has been linked to several other cellular processes that are important to cancer biology. As described in the introduction, both p53 and FoxM1 contribute (in opposite ways) to pluripotency. In embryonal carcinoma cells, FoxM1 is required for the expression of genes like Sox2, Oct4, and Nanog that are important for maintenance of pluripotency (Xie et al., 2010). p53, on the other hand, represses Oct 4 and Nanog (Qin et al., 2007), and reduces the efficiency at which cells can be reprogrammed (i.e. the generation of iPS cells) (Reviewed in (Tapia and Scholer, 2007)). Furthermore, FoxM1 overexpression in keratinocyte progenitor cells prevents their terminal differentiation and leads to clonal expansion of this population with resulting neoplasia. When FoxM1 is overexpressed in differentiated keratinocytes, however, clonal expansion does not occur. Thus, FoxM1 overexpression can contribute to early steps in tumorigenesis (Gemenetzidis et al., 2010).

The mutation of p53 correlates with stem-cell-like properties (Mizuno et al., 2010) and in certain cancer types is thought to be an early event. For example, mutation

of p53 is observed in the early stages of development of many skin cancers, such as squamous cell carcinomas as well as some forms of basal cell carcinomas (Erb et al., 2008). p53 guards against cellular transformation in the skin, as it facilitates cell cycle arrest of skin cells that have undergone DNA-damage events as a result of UV-light exposure. If p53 is mutated in a skin progenitor cell (perhaps through the DNA-damage caused by UV-light), then FoxM1 levels may be deregulated. Further exacerbating the situation is the observation that FoxM1 protein is stabilized by UV light in a Chk2-dependent manner (Tan et al., 2007). As FoxM1 stimulates its own transcription (Halasi and Gartel, 2009), increased levels of FoxM1 protein may facilitate its own mRNA overexpression in the absence of the negative regulation provided by functional p53. Intriguingly, FoxM1 upregulation has in fact been shown to precede squamous cell carcinoma formation (Gemenetzidis et al., 2009). Future work will begin to test this model of the initiation events in skin cancer, but can also be extended to multiple cancers of epithelial origin where p53 has been observed to be mutated in an early stage (e.g breast cancer). Furthermore, both FoxM1 and p53 have been shown to contribute to later events in carcinogenesis as well. As such, loss of p53 at any point during cancer progression may lead to FoxM1 deregulation, or prevent its repression in response to therapy. Such deregulation of FoxM1 may contribute to the undifferentiated state observed in cancer cells.

Alternatively, the deregulation of FoxM1 expression after p53 loss may contribute to other areas of cancer biology. For example, FoxM1 contributes to new blood vessel formation through the induction of VEGF, uPA, uPAR, MMP-2, MMP-9 (Ahmad et al., 2010) (Wu et al.). Furthermore, FoxM1 counteracts oxidative-induced senescence (Li et

al., 2008). On the other hand, p53 has a well defined role in establishing senescence (Qian and Chen, 2010) and has been reported to counteract angiogenesis through various mechanisms (Ravi et al., 2000; Teodoro et al., 2006). Interestingly, p53 restoration in a murine liver cancer model leads to increased both senescence and destruction of neo-vasculature (Xue et al., 2007). Intriguingly, this p53 restoration also led to an approximate 5-fold reduction of FoxM1 mRNA (Wen Xue, personal communication). Thus, the effect of p53 mutation on both angiogenesis and senescence could be evaluated in the context of FoxM1 expression. That is, restoration of p53 in this model on the background of overexpressed FoxM1 would reveal the contribution of p53-dependent FoxM1 repression to such processes.

Both p53 and FoxM1 respond to DNA-damage. As mentioned, FoxM1 protein is stabilized by UV light in a Chk2-dependent manner, and induces the DNA-damage repair genes *Xrcc1* and *Brca2* (Tan et al., 2007). Furthermore, FoxM1 induces Topoisomerase alpha II, the target of many chemotherapeutics that result in DNA-damage (Wang et al., 2009). Thus, it is conceivable that the overexpression of FoxM1 in cancer cells will mediate greater resistance to standard chemotherapeutics that cause DNA-damaging signals. This model would predict that siRNA to FoxM1 in an otherwise p53-null background would cause greater susceptibility to DNA-damaging agents through the inhibition of DNA-repair pathways- future work will address this hypothesis.

Several small molecules have been reported to inhibit FoxM1 levels and activity, including the proteasome inhibitor bortezomib (Velcade) and thiazole antibiotics (thiostreptin and siomycin A) (Bhat et al., 2009a, b; Kwok et al., 2008; Radhakrishnan et al., 2006). The opposing functions of p53 and FoxM1 coupled with our observations that

FoxM1 is a target of p53-mediated repression warrant further study into the possibility of pharmacologically inhibiting FoxM1 in human cancer. As previously discussed, this chemotherapeutic intervention strategy offers the advantage of partially restoring p53 function and inhibiting processes that oppose p53-function in the absence of functional p53. Future work into the *in vivo* inhibition of FoxM1 will reveal whether FoxM1 could represent an “Achilles’ heel” of p53-mutant/inactivated tumors. Furthermore, the elucidation of additional FoxM1 transactivation targets may also elucidate novel targets of p53-mediated repression and novel therapeutic targets.

PVT1

Another strategy to identify targets of p53-mediated repression is to identify non-coding RNAs that are transactivated by p53. Such non-coding RNAs more often than not facilitate downstream repression. For example, microRNAs target mRNAs for degradation, or inhibit their translation. As previously discussed, p53-mediated repression is facilitated by several p53-inducible microRNAs (e.g. miR-34a, miR-145, miR-192, miR-215 and miR-107) as well as the p53-inducible lincRNA-p21. Such non-coding RNAs function to inhibit cell cycle proliferation, cell survival and angiogenesis. In an effort to identify additional pathways and p53-repressed genes, we have focused on the p53-dependent induction of the PVT1 non-coding locus.

As described in Chapter 3, PVT1 encodes a large array of non-coding RNAs. Many PVT1 encoded-RNAs resemble mRNA transcripts, as they are processed from a nascent transcript containing both introns and exons that is subject to regulation by splicing processes in order to produce a final mature transcript. The PVT1 locus is very

large (over 300 kb) and contains at least 9 exons in addition to several alternatively spliced exons. Furthermore, the PVT1 locus encodes a series of 7 microRNA stem-loop structures, 6 of which are annotated in miRBase. These microRNAs are as follows: miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p and miR-1208 (Huppi et al., 2008).

We have confirmed that p53 induces transcription from this locus in response to activation by DNA-damage or Nutlin-treatment. To do so, p53 binds a canonical response element adjacent to miR-1204. In Chapter 3, we presented evidence that miR-1204 may represent a functional target of p53 at this locus. Thus, one aspect of future work will be directed at determining the precise biological functions of miR-1204, as well as deciphering the biological contexts in which miR-1204 regulation is most important.

In order to determine the function of miR-1204, we have overexpressed miR-1204 in a variety of cell lines. In each case, anti-proliferative responses were observed (Chapter 3). In HCT116 cells, cell death ensued. In contrast, ectopic expression of miR-1204 in RKO and Sk-Hep1 cells caused G1 arrest, but not cell death. This difference of phenotypes in different cell lines or different cell types is not without precedence: miR-34a has been shown to contribute to cell cycle arrest, senescence or cell death depending on the cellular context (Hermeking, 2010; Tazawa et al., 2007).

To begin to address the role of miR-1204 in such biological systems, identification of miR-1204 target genes/pathways is of paramount importance. As an experimental starting point, ectopic miR-1204 expression in HCT116 cells (specifically HCT116 DICER^{ex5}) may be followed with gene expression arrays. HCT116 DICER^{ex5}

cells contain a hypomorphic DICER, and thus lower levels of endogenous microRNAs (Cummins et al., 2006). In this setting, ectopic microRNAs give rise to more intense changes in gene expression compared to wild-type DICER cells, facilitating the ease of identifying miRNA gene targets (Linsley et al., 2007). While potentially missing targets that are regulated solely at the level of translation, the overexpression of ectopic miRNAs followed by gene expression arrays (which assay for changes in mRNA levels) has been implemented by many studies in order to identify miRNA target genes, including one study that identified several cell cycle genes as targets of p53-inducible miR-192 and miR-215 (Georges et al., 2008).

An alternative, biochemical strategy to identify miR-1204 targets could also be employed. In this strategy, biotinylated miR-1204 would be used as the bait to pull down endogenous mRNAs. To facilitate specificity, the Flag-tagged Argonaute proteins (key components of the RISC complex) can first be immunoprecipitated to reduce background RNA signals, as microRNAs and target mRNAs should be associated with the RISC complex. After this step, biotinylated microRNAs can be purified using streptavidin beads (Nonne et al., 2010). Any co-purified mRNA should theoretically be a target of miR-1204. This method represents a relatively unbiased approach to target identification, and would identify mRNAs regulated both by degradation and translational inhibition.

Using either methodology to identify miR-1204 targets, these types of analyses will begin to reveal the cellular pathways that are enriched in miR-1204 regulated targets. Such analyses can also be combined with bioinformatics approaches to assess which putative targets of miR-1204 contain the miR-1204 seed sequence. Bioinformatics as a stand alone method, however, has thus far not been particularly useful in the

identification of miR-1204 targets. This is because the most commonly used algorithms either do not make predictions for miR-1204, or do not predict consensus targets. For example, nine different algorithms predict the same gene as a potential miR-34a target, whereas only three algorithms commonly identify a particular mRNA as a target of miR-1204 (data not shown).

As we have observed increased cell death in HCT116 cells, such analyses will help to decipher the exact mechanism by which miR-1204 causes cell death. To do so, we will focus on miR-1204 target genes from the microarray/biochemical/bioinformatic analyses that function in apoptotic/survival pathways. In an attempt to establish such genes as true targets of miR-1204, we will first validate that the endogenous mRNA and protein levels are reduced by ectopic miR-1204. Further, the 3'UTR (containing the seed sequences) of such target genes can be cloned into luciferase reporter assay to assess their regulation by ectopic miR-1204 or endogenous miR-1204 (through the use of microRNA inhibitors/antagomiRs). To confirm the importance of such target genes, these genes can be suppressed using siRNA, or overexpressed in the context of miR-1204 expression. As our data suggests that ectopic miR-1204 stabilizes p53, it is likely that some miR-1204 target genes may in fact function upstream, or even within the context of the p53 pathway. Preliminary data also suggest that the cell death induced by ectopic miR-1204 is at least partially p53-dependent, as HCT116 p53^{-/-} cells are refractory to death by miR-1204 as evidenced by reduced PARP cleavage and reduced sub-G1 content (Figure 4). These findings are not without precedence, as the canonical p53-inducible miR-34a inhibits SIRT1 and thereby increases acetylation of p53, leading to enhanced stability and activity. Furthermore, ectopic miR-34a causes partially p53-dependent cell death

(Yamakuchi et al., 2008), (Figure 4). Thus, miR-1204 is likely to inhibit mRNA targets whose protein products negatively regulate p53 levels or activity.

Future studies will also address the precise regulation of miR-1204 within the context of the PVT1 locus. As described in Chapter 3, p53 preferentially induces transcripts that contain Exon 1B (over 1A). However, the consensus splice site of exon 1B overlaps with the stem-loop structure of miR-1204. Thus, inclusion of exon 1B into spliced PVT1 transcripts could potentially disrupt miR-1204 biogenesis (Figure 5A). As such, it is possible that either miR-1204 or exon 1B is processed out from the nascent PVT1 transcript in a mutually exclusive manner. Yet, our data suggest that both Exon 1B-containing transcripts and miR-1204 expression are simultaneously increased by p53-dependent means. This could be a reflection of a general increase in transcription from this genomic region (i.e. there are enough nascent transcripts to go around).

Alternatively, our preliminary studies suggest another possibility. These studies have revealed a novel version of exon 1B (extended by 63 nt in the 3' direction) expressed in HCT116 cells that contains the entirety of the miR-1204 stem-loop structure (Figure 5B). Nutlin-activated p53 facilitates the induction of two forms of exon 1B, with the slower migrating form representing usage of the extended exon 1B. However, this transcript is also present at basal levels of p53, and can be marginally induced in the absence of p53 by the DNA-damaging agent daunorubicin (data not shown), suggesting that the usage of this extended exon is not entirely dependent upon p53.

Regardless, transcripts containing an extended 1B would allow both splicing events (incorporation of Exon 1B) and subsequent miR-1204 processing. As pre-mRNA splicing events are thought to be coupled with transcription (Chen and Manley, 2009), the

microRNA biogenesis machinery may have to compete with the splicing machinery to process miR-1204 transcripts before the splicing event at Exon 1B occurs. The use of the extended exon 1B transcript may represent a cellular control mechanism that allows extra time and opportunity for microRNA biogenesis of miR-1204 to occur. Furthermore, the identification of this exon extension may have exciting ramifications on the highly abundant fusion transcripts between exon 1B and the immunoglobulin constant region. If extended exon 1B is captured in such fusion transcripts, there is potential for abundant expression of miR-1204.

The function of the PVT1 mature/spliced non-coding RNA as well as the other microRNAs of this locus will also be addressed in future studies. As described previously, PVT1 is translocated, amplified and overexpressed in cancer. The pro-tumorigenic role for PVT1 could be carried out by the microRNAs of this locus, PVT1 ncRNA, or both.

Thus far, we have shown that all primary microRNAs with the exception of miR-1208 are induced by p53. However, we have demonstrated only that mature miR-1204 is induced by p53, and that miR-1207-5p is not. Future experiments will determine if p53 regulates the mature form of any of the other PVT1-encoded microRNAs. As seen in Chapter 3, ectopic expression of miR-1205, miR-1207-5p, and miR-1207-3p all give rise to increased cell death in HCT116 cells. Ectopic miR-1206, on the other hand, does not alter the cell cycle profile or affect cell survival. However, these results must be interpreted with caution as we have not explored if all microRNAs accumulate to similar levels in HCT116 cells. Future work will address the varying, or perhaps complimentary functions, of the PVT1-encoded microRNAs.

Furthermore, PVT1 ncRNA is induced by p53. However, our RT-PCR assays failed to detect any full length transcripts (data not shown), an observation also noted in a recent study of PVT1 in pancreatic cancer (You et al., 2011). Rather, we have identified short transcripts that include various exons. For example, RT-PCR in HCT116 using forward primers in exon 1 and reverse primers in exon 9 reveal transcripts that contain Exon 1,8, and 9, but skip most internal exons (data not shown). However, other RT-PCR experiments using reverse primers within internal exons have revealed that such transcripts are expressed and induced by p53. (e.g. a transcript containing exon 1B,3,4) (Figure 5B). Careful analysis should reveal the spectrum of transcripts induced by p53, as well as the connectivity of the exons (or alternative exons) within such transcripts. After their identification, such transcripts can be cloned and overexpressed in order to assess their function. Furthermore, siRNA to specific endogenous transcripts may reveal their functions. In the immediate future, we plan to overexpress full length sense PVT1 cDNA and antisense PVT1 cDNA in order to ascertain overall function of these transcripts. Such constructs were developed and used to show that PVT1 regulates sensitivity to the chemotherapeutic gemcitabine (You et al., 2011).

Yet another way to begin to understand the function of the PVT1 locus is to analyze expression data sets, focusing on both expression across multiple tissue types and the expression patterns observed in cancer. For example, future studies will attempt to discern which cell types abundantly express the PVT1-encoded microRNAs. Within tumors, the correlation of p53 status and various PVT1-encoded transcripts could be made. Both such analyses will aid in the formulation of hypotheses as to the biological function(s) of PVT1-encoded transcripts and microRNAs.

One such hypothesis is that PVT1-encoded microRNAs function in B-cell differentiation. This is based on the observation that the expression of primary microRNA transcripts of miR-1204 and miR-1206 correlate with the stages of B-cell development; increased expression with increased differentiation. Specifically, late stage B-cells (including plasmacytoma and variant Burkitt's lymphoma cell lines) contain high levels of such microRNAs. In addition, a correlation between high miR-1204 levels and cells that produce the lambda light chain immunoglobulin was noted, leading to the hypothesis that miR-1204 may have a role in the control of immunoglobulin lambda expression, or immunoglobulin light chain expression in general (Huppi et al., 2008). Intriguingly, p53 is also involved in B-cell differentiation and apoptosis (Aloni-Grinstein et al., 1995; MacPherson et al., 2004; Shaulsky et al., 1991). In fact, gamma-irradiation causes accumulation of p53 in pre-B-cells that leads to both apoptosis and differentiation. This differentiation correlates with accumulation of cells at the G2 phase of the cell cycle, and increased immunoglobulin kappa light chain expression (Aloni-Grinstein et al., 1995). Thus, future work will address the hypothesis that p53-induced expression of miR-1204 plays a role in the differentiation of B-cells and immunoglobulin light chain expression.

Future work will also address the hypothesis that p53-dependent induction of miR-1204 leads to cellular senescence. As described in Chapter 3, a recent study found miR-1204 to be the microRNA most induced by the onset of replicative senescence (Marasa et al., 2010). Future studies will address whether this induction of miR-1204 is dependent upon p53. To do so, we will employ siRNA knockdown of p53 in senescent cells, as well as stable cell lines expressing shRNA to p53 that will be grown for

prolonged periods of time to induce to their senescence. Furthermore, we will address the specific contribution of miR-1204 to senescence using microRNA inhibitors, or stable expression of microRNA sponges to divert/capture cellular miR-1204. To date, we have demonstrated that transient miR-1204 expression reduces cell viability of normal human fibroblasts (see Chapter 3), but did not carry these experiments out long enough to determine whether miR-1204 expression alone can induce senescence. To do so, we will stably overexpress miR-1204 precursors (retroviral delivery) to assess if such cells enter replicative senescence at an earlier passage than control infected cells.

Studies into the biological functions of PVT1 should provide deep insight into cancer biology as PVT1 is a site of frequent alteration in tumors. Furthermore, the fact that wild-type p53 regulates this locus suggests that either PVT1 also harbors tumor suppressive properties that are deregulated in cancers, or that p53 has unknown pro-survival functions through the induction of PVT1. Future studies will undoubtedly shed light on this complicated picture, identifying both novel targets of p53-mediated repression and ultimately novel p53-functions.

Overall, the work presented in this dissertation has identified novel targets of p53-mediated regulation. More specifically, Chapters 2 and 3 have focused on the ability of p53 to transactivate genes whose products facilitate downstream repression. The p21 protein contributes to repression of the oncogenic transcription factor FoxM1. Similarly, the PVT1-encoded microRNAs/ncRNAs are likely to inhibit gene expression. Together, these data demonstrate that diverse cellular entities are transcriptionally regulated by p53, and that such entities are likely to mediate p53-dependent processes (Figure 6).

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Figure Legends

Figure 1. p53 represses FoxM1 at the level of transcription. **A.** p53-null H1299 cells were co-transfected with FoxM1-luciferase reporter/p21-luciferase reporter and either ectopic p53 (black bars) or an empty vector control (hatched bars). Relative luminescence is graphed and represents a read-out of transcription. **B.** MCF7 cells (which harbor endogenous wild-type p53) were transfected with either FoxM1-luciferase reporter or p21-luciferase reporter and treated with either DMSO (hatched bars) or Nutlin-3a (10 μ M) (black bars). **C.** HCT116 or HCT116 p53^{-/-} cells were transfected with the FoxM1-luciferase reporter and treated with either DMSO (hatched bars) or Nutlin-3a (10 μ M) (black bars). FoxM1, p53, p21 and Actin levels were detected by immunoblotting.

Figure 2. p53-mediated repression of FoxM1 is not a secondary consequence of G1 arrest. HCT116 cells were treated with either DMSO, Nutlin-3a (10 μ M), or L-mimosine (250 nM). **A.** Levels of FoxM1, p53, p21 and Actin were detected by immunoblotting. **B.** HCT116 cells were stained with propidium iodide to assess their DNA content by FACs analysis. The cell cycle profiles and percentages of cells in G1, S, G2/M were analyzed through the use of Modfit, and are shown for each condition. DMSO represents a negative control treatment. Compared to the negative control, Nutlin causes a decrease in %S and %G1, but an increase in %G2/M. In contrast, L-mimosine causes an increase in %G1 and %S, but a decrease in %G2/M.

Figure 3. Ectopic FoxM1 decreases the percentage of Nutlin-treated U2OS cells with 4N DNA content. U2OS C3 cells contain tetracycline-inducible FoxM1. These cells were pre-treated or not treated with tetracycline 6 hours before the addition of Nutlin-3a (10 μ M). Cells were grown in Nutlin +/- tetracycline for an additional 66 hours. **A.** Relative FoxM1 levels were assessed by immunoblotting. Actin was included as a loading control. **B/C.** U2OS C3 cells were stained with propidium iodide to assess their DNA content by FACs analysis. The cell cycle profiles and percentages of cells in G1, S, G2/M were analyzed through the use of Modfit, and are shown for each condition. The % of cells in G1, G2/M and S-phase are graphed: Nutlin -tet (hatched bars), Nutlin +tet (black bars). Ectopic FoxM1 causes a significant increase in cells with 2N content (G1), and significant decrease in cells with 4N content (G2/M).

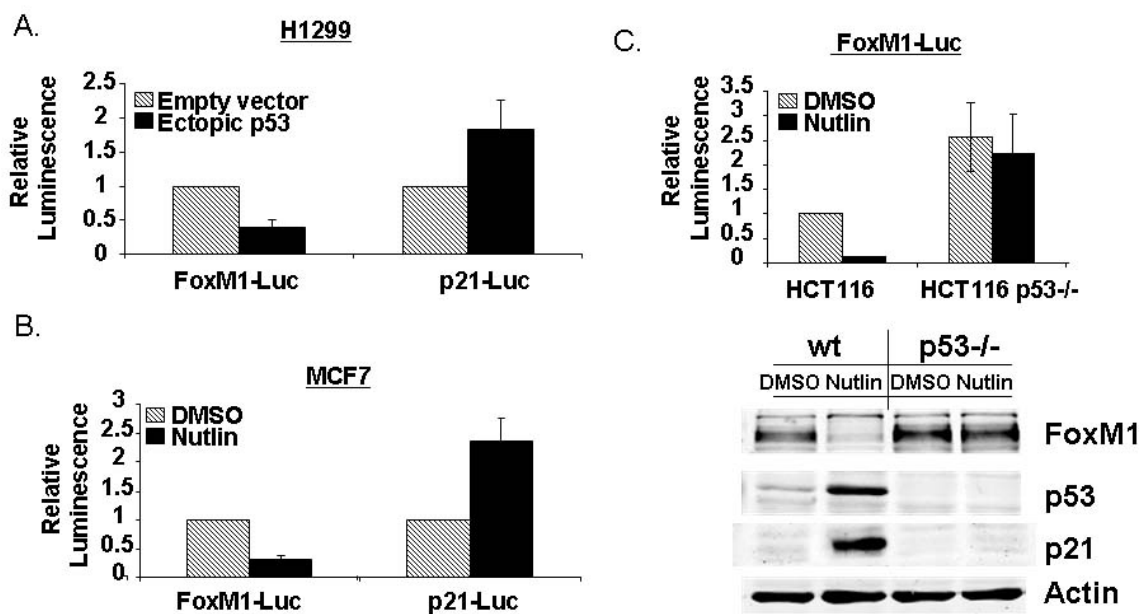
Figure 4. miR-1204 and miR-34a cause partially p53-dependent cell death of HCT116 cells. **A.** HCT116 (hatched bars) or HCT116 p53^{-/-} cells (black bars) were transfected with 20 nmol negative control microRNA mimic (Qiagen All-stars), miR-1204 mimic, or miR-34a mimic. Cells were stained with propidium iodide and subjected to FACs analysis. Cellquest software was used to assess the percentage of cells with Sub-G1 DNA content, indicative of cells undergoing apoptosis. **B.** PARP, cleaved PARP, p53, p21 and Actin levels were detected by immunoblotting.

Figure 5. p53 induces an extended version of PVT1 Exon 1B. **A.** Schematic diagram depicting how PVT1 Exon 1B splicing events may disrupt the biogenesis of miR-1204. As the consensus splice site of exon 1B overlaps the sequence of the miR-1204 “stem-

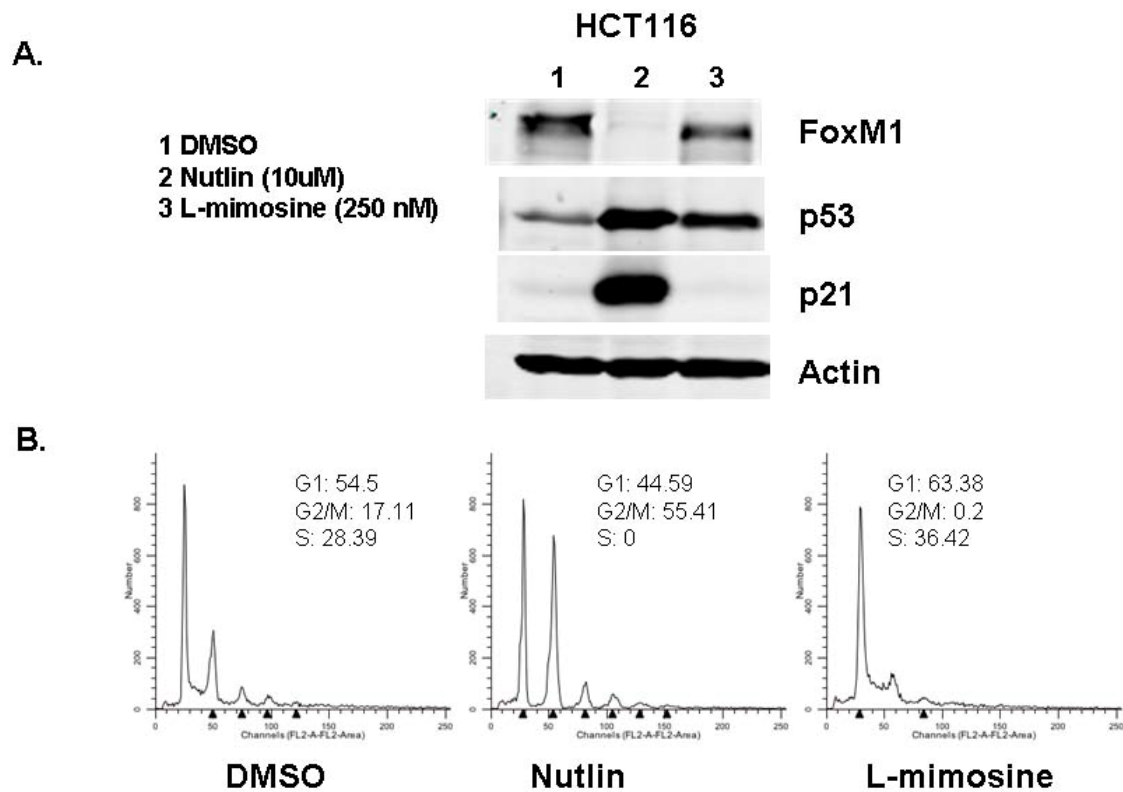
loop,” inclusion of exon 1B into PVT1 mRNA could potentially disrupt miR-1204 processing. **B.** HCT116 and HCT116 p53^{-/-} cells were treated with DMSO or Nutlin-3a (10 μ M). RNA was isolated using the RNeasy mini kit (Qiagen), and cDNA was synthesized with the Quantitect reverse transcription kit (Qiagen). Semi-quantitative PCR was used to amplify PVT1 transcripts (forward primer in exon 1B and reverse primer in exon 4) from this cDNA. The PCR products were separated on an agarose gel and visualized with Ethidium Bromide staining. Semi-quantitative RT-PCR reveals that two distinct transcripts that each contain exon 1B, 3 and 4. The upper band includes a previously unidentified 63 nucleotide sequence extension that encompasses the entire miR-1204 “stem-loop” and thus may allow processing of miR1204 from mature PVT1 mRNA. Both species/bands are induced by p53 after Nutlin treatment. The right panel is a schematic depicting the position of Exon 1B and extended Exon 1B in relation to the miR-1204 stem-loop structure.

Figure 6. p53 induces the expression of diverse cellular entities that facilitate downstream repression of targets involved in cell cycle progression and cell survival.

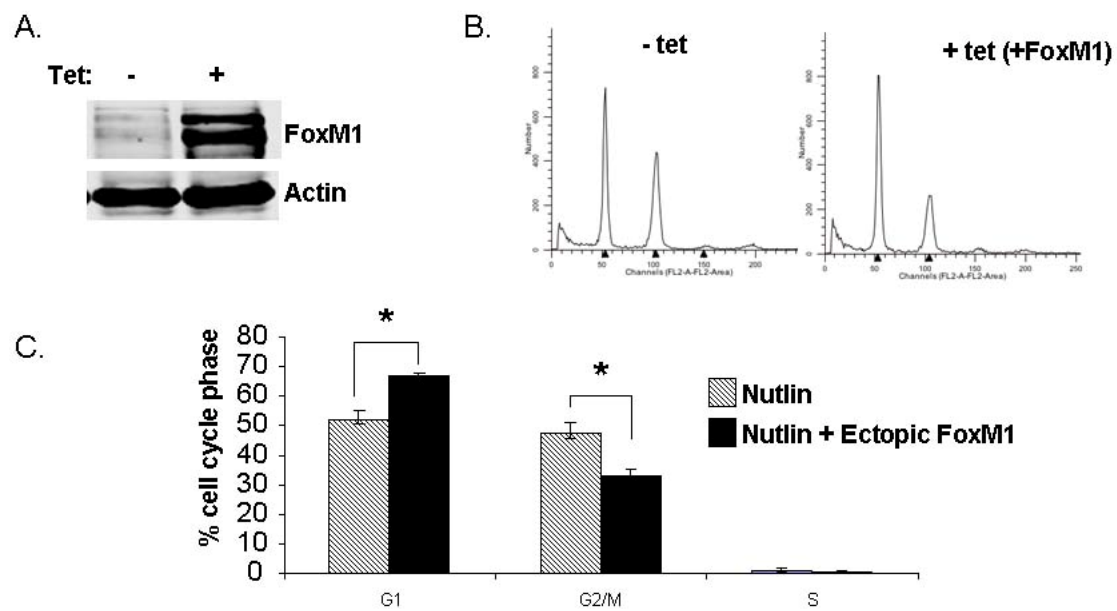
p53 activates the transcription of p21 mRNA, whose protein product represses FoxM1 transcription by activating the Rb-family. p53 also induces the transcription of both miR-1204 and PVT1 ncRNA which potentially function in processes related to both cell cycle arrest and apoptosis.

Luciferase assays: FoxM1-promoter luciferase reporter

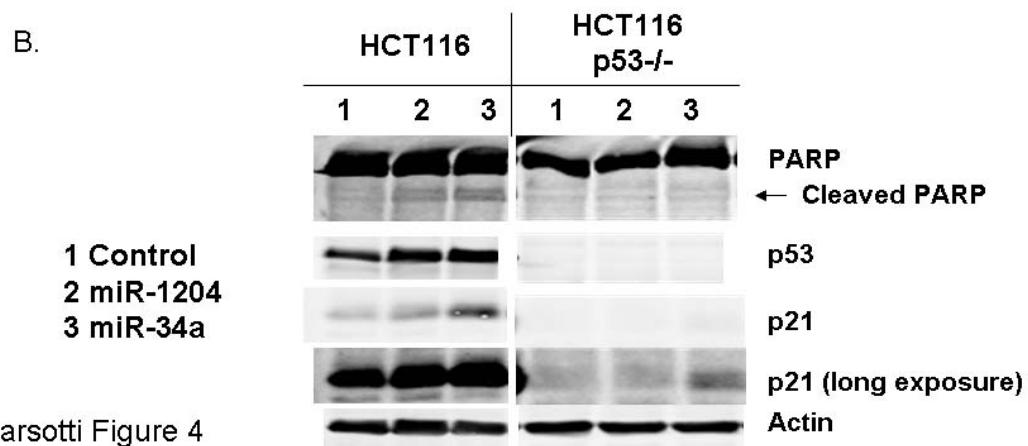
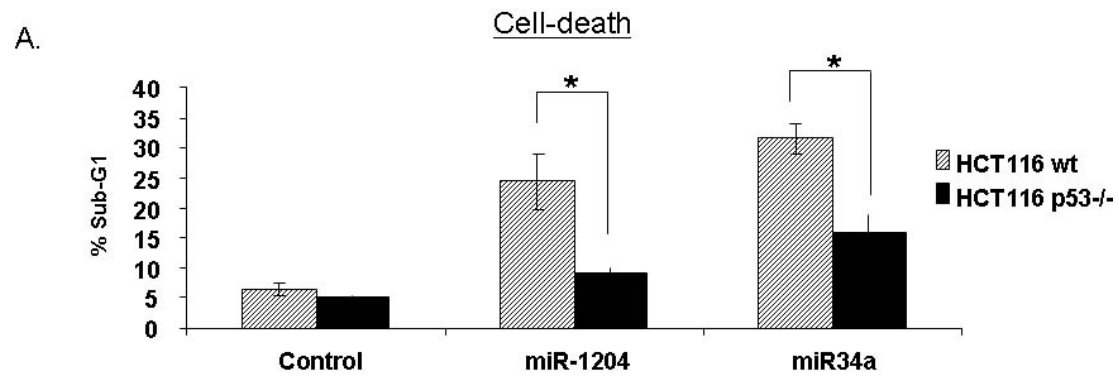
Barsotti Figure 1



Barsotti Figure 2

Nutlin-treated U2OS C3 cells (tet-on FoxM1)

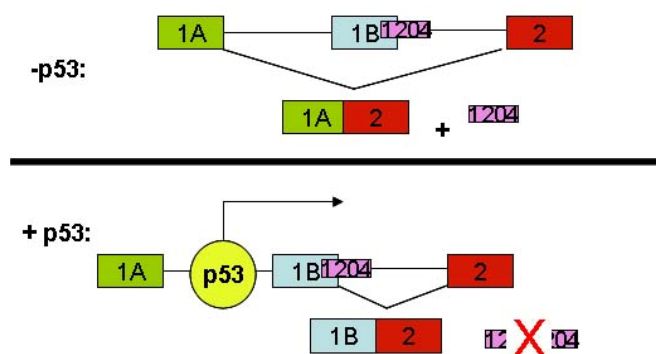
Barsotti Figure 3



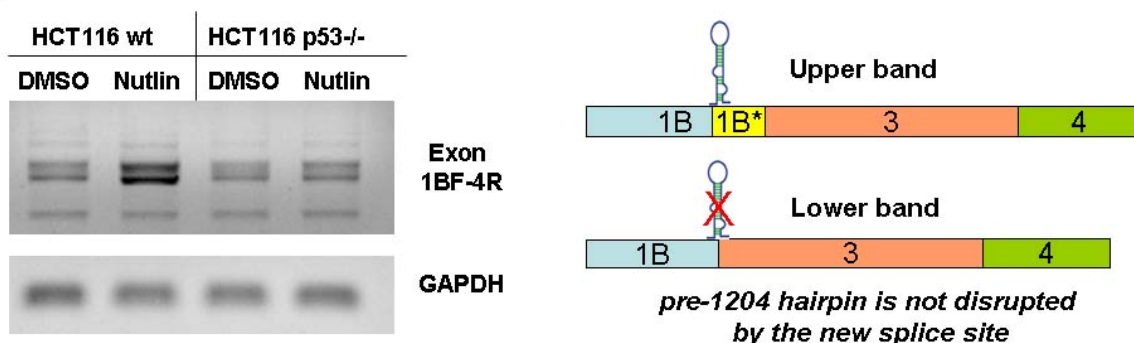
Barsotti Figure 4

A.

Could the use of Exon 1B disrupt miR1204?

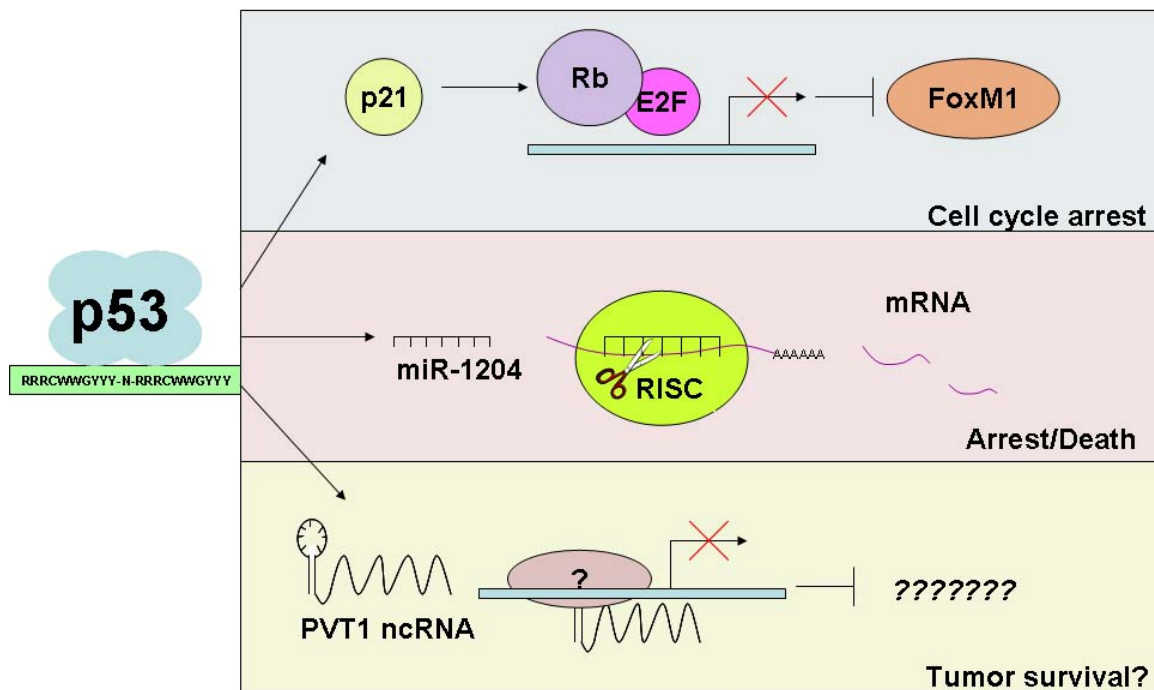


B.



Barsotti Figure 5

Activation for the sake of repression



Barsotti Figure 6